Microbial Degradation of Phenol: A Comparative Study

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By

Satya Sundar Mohanty
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Under the Guidance of

PROF. G. R. SATPATHY
(DEPARTMENT OF BIOTECHNOLOGY & MEDICAL ENGINEERING)

&

DR. H. M. JENA
(DEPARTMENT OF CHEMICAL ENGINEERING)

DEPARTMENT OF BIOTECHNOLOGY & MEDICAL ENGINEERING
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA
ORISSA -769 008, INDIA

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Dedicated to

Mr. Baidhar Mohanty

&

Mrs. Swapna Mohanty

CERTIFICATE

This is to certify that the thesis entitled ***Microbial Degradation of Phenol: A Comparative Study,*** *submitted by* ***Satya Sundar Mohanty*** *to National Institute of Technology, Rourkela is a record of bonafide research work under our supervision and is worthy of consideration for the award of the degree of Master of Technology (Research) of the Institute. The candidate has fulfilled all prescribed requirements and the thesis, which is based on candidate's own work, has not been submitted elsewhere for a degree or diploma.*

Sd-

Co-supervisor

Dr. H. M. Jena
Department of Chemical Engineering

National Institute of Technology
Rourkela - 769008
INDIA

Sd- 07/01/2012

Supervisor

Prof. G. R. Satpathy
Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela - 769008
INDIA

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NOMENCLATURE

Acetyl Co-A	Acetyl Coenzyme A
ANN	Artificial Neural Network
ATCC	American Type Culture Coolection
ATP	Adenine Triphosphate
ATSDR	Agency For Toxic Substances And Disease Registry
BRS	Bovine Respiratory Syncytial Virus
BVD	Bovine Viral Diahorrea
CSP	Clear Soluble Phenol
CTAB	Cetyl Trimethylammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DOE	Design Of Experiment
EDTA	Ethylenediaminetetraacetic Acid
EDX	Energy-Dispersive X-Ray Spectroscopy
EPA	Environmental Protection Agency
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
HCL	Hydrocholoric Acid
IBR	Infectious Bovine Rhinotracheitis Virus
IMTECH	Institute Of Microbial Technology

KCL	Potassium Chloride
K_i	Inhibition Constant
K_s	Half Saturation Coefficient (mg l^{-1} hr^{-1})
mg/L	Milligram Per Liter
$MgCl_2$	Magnesium Chloride
mM	Millimole
MRA	Multiple Regression Analysis
MSM	Mineral Salt Medium
MTCC	Microbial Type Culture Collection
NaCl	Sodium Chloride
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate
NCIM	National Collection Of Industrial Microorganism
O.D	Optical Density
OA	Orthogonal Array
PAA	Polyacrylic Acid
PAAH	Polyacrylamide Hydrazide
PAHs	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
PI3	Parainfluenza3 Virus
pKa	Acid Dissociation Constant
pmol/µl	Picomole Per Microlitre
PPM	Parts Per Million

RNA	Ribonucleic Acid
RPM	Rotation Per Minute
rRNA	Ribosomal Ribonucleic Acid
rs_{max}	Maximum Substrate Consumption Rate (hr^{-1})
S/N	Signal-To-Noise Ratio
SBR	Sequence Batch Reactor
SDS	Sodium Dodecyl Sufate
SEM	Scanning Electron Microscopy
SKIP	Sum Kinetics With Interaction Parameters
Succinyl Co-A	Succinyl Coenzyme A
TCA Cycle	Tricarboxylic Acid Cycle
TCE	Trichloroethane
TE	Tris EDTA Buffer
ToMO	Toluene/O-Xylene Monooxygenase
VP	Voges-Proskauer
μ_{max}	Maximum Specific Growth (hr^{-1})

ABSTRACT

Phenol is one of the most common toxic environmental pollutants that originate mainly from industrial processes. It is a recalcitrant and hazardous compound, which is toxic at relatively low concentration and hence, USEPA has set a limit of 0.1mg/L of phenol as the permissible limit in the water bodies. It must be removed from the environment. The biodegradation methods for the treatment of phenol contaminated wastewater are more effective and less costly. The use of microbial catalysts in the purpose has advanced significantly during the past three decades and it has been found that large numbers of microbes coexist in almost all natural environments, particularly in soils.

Most of the studies were done on the indigenous microbes isolated from the contaminated sites of industrial effluents and waste waters. But often the phenol contamination due to other sources has been overlooked by the scientific community. It is a well known fact that phenol is the major component of most of the disinfectant in recent time. Hence phenol contamination due to hospital wastes and sewage is a common problem in the water bodies located in the nearby areas. In the present study, a novel strain named *Pseudomonas sp.* NBM11 isolated from the soil contaminated with phenol from hospital waste was investigated for its biodegradation potential.

The physiological parameters of the isolate were optimized using the Taguchi method of optimization and it was found that the optimum temperature and pH condition for the microorganism is 30^{o}C and 7 respectively. *Pseudomonas sp.* NBM11 can degrade up to 1000 ppm of phenol completely within 168 hours while it is able tolerate up to a concentration of 1100 PPM and an initial substrate concentration above it inhibits the microbial growth. Biodegradation study of several microorganisms has also been carried

out and the degradation behavior of the isolated strain and the reference microorganisms has been compared. Attempts have been made to enhance the biodegradation potential of the microbe by immobilizing them in calcium alginate beads. It has been observed that the isolated strain is able to degrade up to 1000 ppm of phenol in 144 hrs as free cell and within 48 hrs when immobilized. Similar studies were carried out for rest of the reference microorganisms and it was found that the microbial degradation of phenol is enhanced up on immobilization. Finally the microbe was screened to have a 199bp gene which is responsible for encoding a 60.523 KD polypeptide called phenol hydroxylase P3 component, a component of the enzyme phenol hydroxylase. The presence of this component indicates that the biodegradation activity of the strain is due to the phenol hydroxylase gene and the microbe degrades the phenol via metacleavage pathway.

KEYWORDS: Phenol; biodegradation; *Pseudomonas sp.* NBM11; *Pseudomonas putida*; immobilization; phenol hydroxylase; *Pseudomonas aeruginosa*; *Acinetobacter calcoaceticus*; calcium alginate; *Pseudomonas resinovorans*; *Pseudomonas pictorum*; *Arthrobacter sp.*; disinfectant.

Chapter 1

INTRODUCTION & LITERATURE REVIEW

Chapter 1

Introduction & Literature Review

Water is the most valuable natural resource that exists on our planet. It covers 70% of the earth's surface and without this invaluable compound; the life on the Earth would not exist. This is a widely recognized fact; pollution of water resources is a common problem that is being faced today. With urbanization and extensive industrialization, the pollution of the environment with man-made (synthetic) organic compounds has become a major problem (Ghisalba, 1983). The natural water bodies are contaminated by various toxic pollutants. The demand for clean and drinking water is becoming a pressing concern day by day due to fast depletion of the freshwater resources since many sources of water bodies have been exhausted because of increasing world population and others are likely to be contaminated. Hence the crisis of fresh water is being faced all over the world.

The industrial and domestic activities have polluted the surface water as well as ground water to a greater extent (Armour, 1991). Wastewater from domestic or industrial use contains waste products and severe environmental pollutants that are most often liquid or solids and are biological, chemical or radioactive. In addition to having adverse health implications, wastewater contamination also has natural and ecological effects. Toxic wastes are being released into the environment, causing extensive environmental contamination such that many of our natural water reserves are damaged beyond repair (Ollis, 2000). Due to discharge of toxic effluents long-term consequence of exposure can cause cancer, delayed nervous damage, malformation in urban children, mutagenic changes, neurological disorders etc (Govindarajalu, 2003). Nevertheless, to ensure sustainable quality of life the environmental impact of these activities must be minimized. While conservation and better utilization of resources have the greatest influence on sustainability of the planet; reduced generation, improved treatment technology and utilization of wastes are the best techniques for the maintenance of the environmental quality.

When the effluents due to various domestic and industrial activities are discharged into the water bodies, aquatic environment get exposed to various toxic compounds present in them. Some of these compounds are not only toxic but only partly, or barely, biodegradable. The microorganism degrades these compounds by which the dissolved oxygen content of the water gets depleted. This depletion below the critical level causes serious damage to the aquatic life.

1.1 XENOBIOTICS

Xenobiotic compounds are man-made chemicals that are present in the environment at high concentrations and are highly toxic in nature. These compounds normally have unusual chemical or physical properties that make them refractory to biodegradation. The principal xenobiotics include drugs, pesticides, carcinogens and various compounds that have been introduced into environment by artificial means, which resist to normal decomposition and persist for longer time in the environment.

Among all the toxic compounds, phenol and its substituent phenolic compounds contribute a remarkable adverse impact to the environment. These are major xenobiotics, which are often found in the effluents discharged from the industries such as paper and pulp, textiles, gas and coke, fertilizers, pesticides, steel and oil refineries etc., (Ghadi and Sangodkhar, 1995; Mahesh and Rama, 1999). During the last two decades, phenolic compounds have become the subject of intense research in the preservation of our environment. The US Environmental Protection Agency (EPA, 1979) had classified the Phenolic compounds as high priority pollutants due to their extensive impact on the deterioration of the water environment.

1.2 PHENOL

Phenol is the common name of hydroxybenzene, an aromatic compound having one hydroxyl group attached to the benzene ring. Phenol has also been called carbolic acid, phenic acid, phenylic acid, phenyl hydroxide or oxybenzene (Nair et al., 2008). Phenol is the basic structural unit for a variety of synthetic organic compounds. It is a white crystalline solid which is soluble in most organic solvents (ATSDR, 2008; EPA, 1979). It is moderately volatile at room temperature (evaporates more slowly than water) and quite flammable

(Calabrase and Kenyon, 1991). It has a very strong odor (acrid odor) with an odor threshold of 0.04 PPM (Amoore and Hautala, 1983) and a sharp burning taste. Phenol is a weak acid and in its ionized form, it is very sensitive to electrophilic substitution reactions and oxidations. The chemical and physical properties of phenol have been enlisted in table 1.1.

Phenol is produced both naturally and synthetically by chemical processes. Naturally, phenol has been extracted from coal tar distillation. Synthetically, cumene oxidation accounts for 95% of phenol production worldwide. The industries like leather, paint, pharmaceutical, coking plant petrochemical, oil refinery, plastic, explosives, steel, pesticides etc and disinfectants uses phenolic and its derivative compounds as their products and raw materials (Busca et al., 2008).

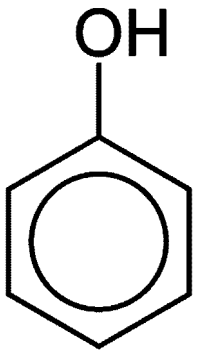

FIG 1.1: STRUCTURE OF PHENOL.

TABLE 1.1: CHEMICAL AND PHYSICAL PROPERTIES OF PHENOL

PROPERTY	PHENOL
Formula	C_6H_5OH
Molecular weight (g/mol)	94.14
Water solubility (g/L at 25 ºC)	87
Melting point (ºC)	43
Boiling point (ºC)	181.8
Auto ignition temperature	715 ºC

Flash point (open cup)	87 ºC
pKa	9.89 X 10^{-10}

1.2.1 USES OF PHENOL

As a pure substance, phenol finds its application in the production of slimicides, disinfectants, antiseptics and medicinal preparations such as ear and nose drops, mouthwashes and sore throat lozenges (ATSDR, 2008). Phenol is used for the preparation of some cream and shaving soap for its germicidal and local anesthetic properties, in veterinary medicine as an internal antiseptic and gastric anesthetic, as a peptizing agent in glue, as an extracting solvent in refinery and lubricant production, as a blocking agent for blocked isocyanate monomers, as a reagent in chemical analysis and as a primary petrochemical intermediate.

Industrially phenol is used for the production of phenolic resins like phenol–formaldehyde resins (Bakelite) which are low-cost thermosetting resins applied as plywood adhesive, construction, automotive and appliance industries. Even for the production of various epoxy resins phenol acts as the precursor compound. By reaction with acetone it may also be converted into bisphenol A, a monomer for epoxy-resins. It is also used to produce cyclohexanone and cyclohexanone–cyclohexanol mixtures by selective catalytic hydrogenation. Cyclohexanone is later converted into its oxime and further to caprolactame, the monomer for nylon 6. The mixture cyclohexanone–cyclohexanol is oxidized by nitric acid to adipic acid, one of the monomers for the production of nylon-6,6. Phenol is also used to produce polyphenoxy and polysulphone polymers, corrosion-resistant polyester and polyester polyols.

Phenol is also a building block for the synthesis of pharmaceuticals, such as, e.g., aspirin (Busca et al., 2008). Phenol is used along with chloroform (a commonly-used mixture in molecular biology for DNA & RNA purification from proteins) and also used for cell disruption and lysis purpose (Sambrook et al., 2000).

1.2.2 PHENOL AS DISINFECTANT

Phenol is probably the oldest known disinfectant introduced by Lister as "carbolic acid". Today disinfectants are widely used in the health care, food and pharmaceutical sectors to prevent unwanted microorganisms from causing disease. Phenolic disinfectants include O-Syl, Matar, Septicol, Hexachlorophene, Environ, One-Stroke, Lysovet, Tek-Trol, Lysol, Pantek, Discan, Pine-sol and Staphene. Phenol-based disinfectants such as Wex-cide, ProSpray, and Birex are germicidal, fungicidal, virucidal, and tuberculocidal i.e. these are effective against many bacteria, fungi and some viruses which are available in moderate cost (Thiel, 1999). Phenols are effective especially against gram positive bacteria and enveloped viruses which include BRS, BVD, Coronavirus, IBR, Leukemia, PI3, Pox, Rabies and Stomatitis virus.

Phenolic compounds are used as intermediate level disinfectants used to treat non critical medical devices which pose the lowest risk of transmission of infection, usually contact only intact skin (Ritcher, 2011).They retain more activity in the presence of organic material than iodine or chlorine-containing disinfectants. These are commonly used in commercial animal production units including hatchery and equipment sanitation, and footbaths (Jeffrey, 1997). Phenolic disinfectants (including cresols and pine oil) are generally safe, but prolonged exposure to the skin may cause irritation.

1.2.3 TOXICITY OF PHENOL

Phenol is a major pollutant included in the list of EPA (1979) as reported by Agarry et al. (2008). Phenol is toxic even at low concentrations and the toxicity of phenols for microbial cells has been investigated (Keweloh et al., 1990; 1998; Kahru et al., 2002). Therefore, the environmentally unacceptable pollution effects of the wastes have been reported worldwide (Ruiz-Ordaz et al., 2001) and the adverse effects of phenol on health are well documented (Calabrase and Kenyon, 1991; Sax, 1984).

Acute exposure of phenol causes disorders of central nervous system. Acute exposure to phenol by the oral route, leads to damage in blood, liver, kidney and cardiac toxicity including weak pulse, cardiac depression and reduced blood pressure. Ingestion of 1

g phenol is reported to be lethal for humans (Kumaran and Paruchuri, 1996). Hypothermia, myocardial depression, burning effect on skin, irritation of the eyes, also it causes gastrointestinal disturbance are some of the effects reported by the researchers (Tziotzios et al., 2005; Chakraborty et al., 2010). Moreover, consumption of water containing high phenolic compounds may lead to cancer as it is a susceptible carcinogen. The exposure of phenol and its derivative compounds to human and animals causes liver and kidney damage, central nervous system impairment, diarrhea and excretion of dark urine (Olujimi et. al., 2010; Khare, 2011).

Due to these adverse health effects of phenolics, as per the rules of World Health Organization the maximum permissible level for phenol in environment is 0.1 mg/l (Kumaran and Paruchuri, 1996; Nuhoglu and Yalcin, 2005; Saravanan et al., 2008).

1.2.4 ENVIRONMENTAL POLLUTION CAUSED BY PHENOLIC WASTE

Phenol and its substituent compounds are the characteristics pollutants in the waste water generated from crude oil refineries, ceramic plants, steel plants, coal conversion processes, manufacturing units of phenolic resins, pesticides and explosives, *etc*. Table 1.2 enlists the various industrial operations and the concentration of the phenol in the effluent generated from them.

TABLE 1.2 PHENOL CONCENTRATIONS IN INDUSTRIAL EFFLUENTS (BUSCA ET AL. 2008)

Industry	**Phenol Concentration (mg L^{-1})**
Coking operations	28 – 3900
Coal processing	9 – 6800
Petrochemicals	2.8 – 1220
Pulp and paper	0.1 - 1600
Gas production	4000
Refineries	6 – 500
Pharmaceuticals	1000
Benzene manufacturing	50

1.3 TREATMENT METHODS FOR THE REMOVAL OF PHENOLIC WASTES

In view of the wide prevalence of phenols in different wastewaters and their toxicity to human and animal life even at low concentrations, it is extremely necessary to remove them before discharge of wastewater into water bodies Therefore, it is very important to employ appropriate strategies of wastewater treatment in order to counterbalance these growing environmental problems. Several treatment technologies have been employed in this regard (Klein and Lee, 1978; Anselmo and Novais, 1992; Koyama et al., 1994; Mokrini et al., 1997; Chan and Fu, 1998; Danis et al., 1998; Reardon et al., 2000; Backhaus et al., 2001; Goncharuk et al., 2002; Ajay et al., 2004). The applied treatment, which could be a single treatment or a combination of these treatments, must guarantee the removal of phenol to allowable discharge limits. The choice of treatment depends upon the concentration, and volume of the effluent treated and cost of the treatment.

1.3.1 PHYSICO-CHEMICAL METHODS FOR REMOVAL OF PHENOL

A variety of treatment methods, such as adsorption, wet oxidation, chemical oxidation etc have been used for removal of phenols from aqueous solutions. Several treatment methods that are available for treating the phenolic waste include granular activated carbon processes and reverse osmosis, anaerobic processes, the electro Fenton method, and combined applications of flotation and coagulation processes, stripping and oxidation.

I) ION EXCHANGE

The removal of an ion from an aqueous solution by replacing another ionic species is the basic principle of Ion exchange method of xenobiote removal. Natural and synthetic materials which are specially designed to enable ion exchange operations at high levels are used to perform this ion exchange for removal of organic and inorganic pollutants for purification and decontamination of industrial effluent. The main features of the ionic resins include properties such as like adsorption capacity, porosity, density etc (Zorpas et al., 2010). Caetano et al., (2009) reported to use two different types of ion exchange resins, Dowex XZ (strong anion exchange resin) and AuRIX 100 (weak anion exchange) for

efficient phenol removal. They reported that both the ion exchange resins showed maximum phenol removal under alkaline conditions.

II) ADSORPTION

Adsorption is a widely used wastewater treatment method for colour, heavy metals and other inorganic and organic impurities present in the industrial effluents as stated by Al-Rekabi et al., (2007) and Patel and Vashi., (2010). Adsorption of phenol onto activated carbon is a widely studied treatment method because of the affinity of phenols for the active surface of carbon (Garcia-Araya et al., 2003).Mechanisms involved in the adsorption process mainly focus on the selection of the adsorbent material like their particle size surface area and porosity etc (Gardea-Torresdey et al., 2004). Kyuya et al. (2004) reported pore size distribution and surface area of the activated carbon are two important factors that affect the adsorption of phenol. Because of the high cost involved in the usage of activated carbon, materials basically obtained from low-cost agricultural wastes; activated carbon prepared from various raw materials such as sawdust, nut shells, coconut shells etc were been used as adsorbent (Zawani et al.,2009). These adsorbents are basically used for the effective removal and recovery of phenolic pollutants from wastewater streams (Basso et al., 2002; Park et al., 2006).

III) CHEMICAL OXIDATION

Chemical treatment involves the use of chemical agents to completely destroy or convert the contaminants to harmless or less toxic compounds, or intermediates that can be further degraded by microorganisms (Hamby D. M., 1996). Chemical oxidation of organic pollutants especially phenol is a promising alternative when wastewater contains non-biodegradable and/or toxic contaminants and also when the contaminant concentration is high. The chemical agents used are normally strong oxidants. The most commonly used oxidants that initiate the oxidation reactions include hydrogen peroxide which is widely used for this purpose (Dias-Machado et al., 2006 ; Ksibi, 2006).

1.3.2 LIMITATIONS OF THE ABOVE WASTEWATER TREATMENT TECHNIQUES

The physico-chemical treatment technologies discussed above found to have inherent drawback owing to the tendency to form secondary toxic intermediates and also proven to

be costly (Klein and Lee, 1978; Talley and Sleeper, 1997). These processes are high energy consuming, non economic and release effluents and waste waters which requires further treatment and thus are alarming for the environment.

The main drawback with ion exchange method is the high cost of the ion exchange resins and each resin must be selectively removes one type of contaminant only. Caetano et al., 2009 reported that the phenol is removed by the ion exchange resins only in the alkaline medium while the maximum phenol removal was obtained by the non functionalized resin in acidic medium. Moreover recovery of these ion exchange resins was tasking process. Further ion exchange is also highly sensitive to pH of the solution (Saparia et al., 1996; Liotta et al., 2009).

In adsorption, phenol in the wastewater is selectively transferred into the solid phase (adsorbent) instead of eliminating it from the wastewater. It once again produces a large amount of solid waste, which further requires a safe disposal. As mentioned earlier, use of activated carbon is not cost effective as high cost factors are associated with the recovery of activated carbon particles from the treated waste water (Banat et al., 2000).

There are many disadvantages associated with the chemical oxidation process like the high cost of the chemicals, emission of various harmful by products, it creates hazardous constituent like secondary effluent problem along with the production of harmful gases (Jena et al., 2005). In case of chemical oxidation of phenol, various oxidizing agents such as hydrogen peroxide, fenton's reagents etc. are used. Hydrogen peroxide when used alone has low reactivity and causes incomplete oxidation of many organic contaminants (Kamenev et al., 1995; Ikehata and Gamal El-Din, 2006).

Hence the development of technology that emphasizes detoxification and degradation of phenol without the above mentioned drawbacks has become the focus of the research. Biological treatment with pure and mixed microbial strains is considered to be an attractive and efficient alternative for the treatment of contaminated wastewaters containing recalcitrant substances such as phenolics since it produces no toxic end products and it is cost effective (Monteiro et al., 2000; Banerjee et al., 2001; Abuhamed et al., 2004; Kumar et al., 2005; Rodriguez et al., 2006).

1.3.3 BIODEGRADATION

Biodegradation is the breakdown of complex and possibly toxic organic contaminants to non-toxic and simpler elements by microbial activity. These contaminants can be considered as the microbial food source or substrate. Biodegradation of any organic compound can be thought of as a series of biological degradation steps or a pathway that ultimately results in the oxidation of the parent compound that often results in the generation of energy. Microorganisms have the capability of degrading all naturally occurring compounds; this is known as the principle of microbial infallibility (Alexander, 1965). However, biodegradation is limited in the number of toxic materials it can handle, but where applicable, it is cost effective (Atlas and Unterman, 1999).

1.3.3.1 ADVANTAGES OF BIODEGRADATION

Biodegradation is being preferred over the other conventional treatment methods of phenol due to its potential to degrade phenol completely and overcome the disadvantages posed by other processes .It produces no harmful end products, cost effective and most importantly maintains phenol concentration below the toxic limit. The microbes break down phenol completely and utilize it in the TCA cycle for energy production.

1.4 MICROORGANISMS IN THE BIODEGRADATION OF PHENOL

The focus on the microbial degradation of phenols in recent years has resulted in the isolation, culture, adaptation and enrichment of a number of microorganisms that can grow on the compound as a sole carbon and energy source. Phenol is an antimicrobial agent; many of the microbes are susceptible to this compound. However, there are some microbes, which are resistant to phenol and have the ability to degrade phenol.

The wide variety of microorganisms that can aerobically degrade phenol include pure bacterial cultures such as: *Acinebacter Sps.* (Paller et al., 1995; Abd-El-Haleem et al., 2003), *Alcaligenes eutrophus* (Leonard and Lindley, 1998), *Arthrobacte*r (Baradarajan et al., 1995), *Bacillus stearothermophilus* (Buswell, 1975), *Nocardioidess* (Cho et al., 2000), *Pseudomonas aeruginosa* (Oboirien et al., 2005; Ojumu et al., 2005), *Pseudomonas cepacia G4* also known as *Burkholderia cepacia G4* (Folsom et al., 1990; Muller, 1994; Schroder et al., 1997; Solomon et al., 1994), *Pseudomonas fluorescens* (Oboirien et al., 2005; Ojumu et

al., 2005), *Pseudomonas pictorum* (Annadurai et al., 2000), *Pseudomonas putida* (Bettman and Rehm, 1984; Gotz and Reuss, 1997; Hill and Robinson, 1975; Hinteregger et al., 1992; Reardon et al., 2000; Yang and Humphrey, 1975), *Pseudomonas resinovorans* (Dikshitulu et al., 1993; Yang and Lee, 2007), *Ralstonia eutropha* (Leonard et al., 1999), while *Desulfobacterium phenolicum sp.* (Bak and Widdel, 1986) can anaerobically degrade phenol.

Moreover, some yeast such as *Candida tropicalis* (Ehrhardt and Rehm, 1985), *Fusarium flocciferium* (Anselmo et al., 1985; Mendoca et al., 2004) and *Trichosporon cutaneum* (Gaal and Neujahr, 1980; Yang and Humphrey, 1975) are also capable of degrading phenol.

Amongst all the microorganisms listed above genus Pseudomonas comprises an important group of bacteria with environmental application in bioremediation and biological control (Agarry et al., 2008). In Pseudomonads, many of its induced enzymes are non-specific and its metabolic pathway contains a high degree of convergence. The convergence of catabolic pathways allow for the efficient utilization of a wide range of growth substrates while the non specificity of the induced enzymes allows for the simultaneous utilization of several similar substrates without an excess of redundant genetic coding for enzyme induction (Hannaford and Kuek, 1999; Monteiro et al., 2000; Banerjee et al., 2001; Abhuhamed et al., 2004; Kumar et al., 2005; Nuhoglu and Yalcin, 2005; Karigar et al., 2006; Rodriguez et al., 2006).

Microorganisms are able to adapt to the presence of toxic organic compounds by using a whole cascade of adaptive mechanisms. Among the adaptive mechanisms, changes in the fatty acid composition of membrane lipids are the most important reactions of bacteria to membrane-active substances (Neumann et al; 2004). One adaptive mechanism enabling several *Pseudomonas* strains to grow in the presence of membrane-disrupting compounds is the isomerization of *cis*unsaturated fatty acids to *trans*-unsaturated fatty acids. This mechanism could also be found in *Pseudomonas* sp. Strain ADP. The extent of the isomerization, usually expressed as the *trans/cis* ratio of unsaturated fatty acids, apparently correlates with the toxicity of organic compounds. Additionally, a mutual dependence was found between the activation of this system and the induction and activation of other stress

response mechanisms. Therefore, an increase in the *trans/cis* ratio can be used as an indicator of environmental stress.

Kotturi et al. (1991) have studied cell growth and phenol degradation kinetics at 10°C for a psychrotrophic bacterium, *Pseudomonas putida* Q5. The batch studies were conducted for initial phenol concentrations, ranging from 14 to 1000mg/1. The experimental data were fitted by non-linear regression to the integrated Haldane substrate inhibition growth rate model. The values of the kinetic parameters were determined. Compared to mesophilic pseudomonads previously studied, the psychrotrophic strain grows on and degrades phenol at rates that are ca. 65-80% lower. However, use of the psychrotrophic microorganism may still be economically advantageous for waste-water treatment processes installed in cold climatic regions, and in cases where influent waste-water temperatures exhibit seasonal variation in the range 10-30 ° C.

Van Schie et al. (1998) isolated and characterized three novel nitrate-reducing microorganisms that are capable of using phenol as a sole source of carbon from anaerobic sediments obtained from three different geographic locations. The three strains were shown to be different from each other based on physiologic and metabolic properties. Even though analysis of membrane fatty acids did not result in identification of the organisms, the fatty acid profiles were found to be similar to those of *Azoarcus* species. Sequence analysis of 16S ribosomal DNA also indicated that the phenol-degrading isolates were closely related to members of the genus *Azoarcus*. The results of this study add three new members to the genus *Azoarcus*, which previously comprised only nitrogen-fixing species associated with plant roots and denitrifying toluene degraders.

Balan et al. (1999) used *Pseudomonas pictorum* (NICM-2077), an effective strain in the biodegradation of phenol. The microorganism was grown on various nutrient compounds which protect it while confronting shock loads of concentrated toxic pollutants during waste water treatment. The effect of glucose, yeast extract, $(NH_4)_2SO_4$ and NaCl on phenol degradation has been investigated and an Artificial Neural Network (ANN) Model has been developed to predict degradation. The network model was then compared with a Multiple Regression Analysis model (MRA) arrived from the same training data.

Santos et al. (2004) isolated thirty filamentous fungal strains from effluents of a stainless steel industry (Minas Gerais, Brazil) and tested for phenol tolerance. Fifteen strains of the genera *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp. and *Graphium* sp. tolerants up to 10 mM of phenol were selected and tested for their ability to degrade phenol. Phenol degradation was a function of strain, time of incubation and initial phenol concentration. FIB4, LEA5 and AE2 strains of *Graphium* sp. and FE11 of *Fusarium* sp. presented the highest percentage phenol degradation, with 75% degradation of 10mM phenol in 168 hours for FIB4. A higher starting cell density of *Graphium* sp. FIB4 lead to a decrease in the time needed for full phenol degradation and increased the phenol degradation rate. All strains exhibited activity of catechol 1,2-dioxygenase and phenol hydroxylase in free cell extracts obtained from cells grown on phenol, suggesting that catechol was oxidized by the *ortho* type of ring fission.

Kumar et al. (2005) carried out biodegradation experiments with phenol and catechol using *Pseudomonas putida* MTCC 1194. The bacterial strain used for the degradation experiments were acclimatized with phenol and catechol up to a concentration of 1000 mg/L and 500 mg/L respectively. They observed that the initial phenol concentration of 1000 mg/L and the initial catechol concentration of 500 mg/L were fully degraded in 162 hours and 94 hours, respectively. Both phenol and catechol exhibited inhibitory behavior and the culture growth kinetics were correlated with Haldane's inhibitory growth kinetic model. They also observed that the bacterial culture died when the initial concentration of phenol and catechol were above 1200 mg/L and 600 mg/L, respectively.

Arutchelvan et al. (2006) have isolated and identified A strain of *Bacillus brevis*. Phenol biodegradation in a batch reactor was studied using the pure culture of *B. brevis*. The isolated strain was optimized for various environmental conditions and the biodegradation of phenol was highest at pH 8.0, 5% (v/v) of inoculum size and without any co-substrate. The biokinetic parameters of biodegradation according to Haldane's equation was determined and was found to be μ_{max} = 0.026–0.078 h^{-1}, K_s = 2.2–29.31 mg/l, K_i = 868.0–2434.7 mg/l. These values are specific for this organism and we have compared with literature for pure or mixed cultures degrading phenol.

Yang and Lee (2007) isolated two strains pure phenol-degrading strains from enriched mixed cultures and identified as *Pseudomonas resinovorans* strain P-1 and Brevibacillus sp. strain P-6. The optimum growth temperatures for P. resinovorans and Brevibacillus sp. were 31 and 39 ^{o}C, respectively. P. resinovorans could degrade phenol completely within 57.5 hours, when the initial phenol concentration was lower than 600mg l^{-1}. If the initial phenol concentration was lower than 200 mg l^{-1}, Brevibacillus sp. could remove phenol completely within 93.1 hours. It was obvious that the phenol-degrading ability of P. resinovorans was much better than that of Brevibacillus sp. The metabolic pathway for P. resinovorans phenol degradation was assigned to the meta-cleavage activity of catechol 2,3-dioxygenase.

Karigar et al. (2006) studied the ability of *Arthrobacter citreus*, isolated from a hydrocarbon contaminated site, to consume phenol as the sole carbon source. The phenol degradation studies in their work showed that complete degradation of the compound occurred within 24 hours. The organism metabolized phenol with a maximum initial concentration of 22 mM, whereas higher levels were inhibitory.

Jiang et al. (2007) isolated a strain of *Alcaligenes faecalis* from activated sludge collected from a municipal gasworks. The phenol biodegradation tests showed that the phenol-degrading potential of *A. faecalis* related greatly to the different physiological phases of inoculum. The maximum phenol degradation occurred at the late phase of the exponential growth stages, where 1600 mg L^{-1} phenol was completely degraded within 76 hours. *A. faecalis* secreted and accumulated a vast quantity of phenol hydroxylase in this physiological phase, which ensured that the cells could quickly utilize phenol as a sole carbon and energy source. In addition, the kinetic behavior of *A. faecalis* in batch cultures was also investigated over a wide range of initial phenol concentrations (0–1600 mg L^{-1}) by using Haldane model. It was clear that the Haldane kinetic model adequately described the dynamic behavior of the phenol biodegradation by the strain of *A. faecalis*.

Agarry et al. (2008) studied the bioremediation potential of an indigenous *Pseudomonas fluorescence* in batch culture using synthetic phenol in water in the concentration range of (100 –500) mg/L as a model limiting substrate. The effect of initial phenol concentration on the degradation process was investigated. Phenol was completely

degraded at different cultivation times for the different initial phenol concentrations. Increasing the initial phenol concentration from 100 mg/L to 500 mg/L increased the lag phase from 0 to 66 hours and correspondingly prolonged the degradation process from 84 hours to 354 hours. There was decrease in biodegradation rate as initial phenol concentration increased. Fitting data into Monod kinetic model showed the inhibition effect of phenol. The kinetic parameters have been estimated up to initial phenol concentration of 500 mg/ L. The rs_{max} decreased and Ks increased with higher concentration of phenol. The biokinetic constants estimated using Haldane model showed good potential of the *Pseudomonas fluorescence* and the possibility of using it in bioremediation of phenol waste effluents.

Cordova-Rosa et al. (2009) reported about the time-course performance of a phenol-degrading indigenous bacterial consortium, and of *Acinetobacter calcoaceticus* var. *anitratus*, isolated from an industrial coal wastewater treatment plant. The bacterial consortium was able to survive in the presence of phenol concentrations as high as $1200 mgL^{-1}$ and the consortium was faster in degrading phenol than a pure culture of the *A. calcoaceticus* strain. A high phenol biodegradation (above 95%) by the mixed culture in a bioreactor was obtained in both continuous and batch systems, but when test was carried out in coke gasification wastewater, no biodegradation was observed after 10 days at pH 9–11 for both pure strain or the isolated consortium.

Shourian et al. (2009) isolated a potent phenol-degrading bacterium, assigned *Pseudomonas* sp. SA01 from pharmaceutical disposal wastewaters plant. According to biochemical characteristics and 16S rRNA sequence analysis, the isolate was identified as *Pseudomonas* sp. The isolated strain started to degrade 0.7 g/l of phenol after an initial very short lag phase, and phenol decomposition was then rapidly completed within 30 hours. *Pseudomonas* sp.SA01 was able to degrade phenol in concentrations up to 1 g/l. Higher phenol concentrations (>1 g/l) had a significant inhibitory effect on bacterial growth. The optimum degradation pH value was found to be 6.5. Addition of mannitol and casein as auxiliary carbon and nitrogen sources enhanced the rate of phenol removal to as low as 20 hours. Based on the absorption spectra of catechol bioconversion of phenol-grown cells, it was concluded that the SA01 strain metabolizes the phenol via a meta-cleavage pathway.

Li et al. (2010) investigated the growth kinetics of a psychrotroph, *Pseudomonas putida LY1*, while growing on phenol as a sole carbon and energy source. This bacterium could completely biodegrade 200 mg/L phenol across a temperature range from 2.5 to 35^{o}C, with an optimum temperature of 25^{o}C. High initial phenol concentrations (800 mg/ml) were inhibitory to bacterial growth. At lower concentrations of phenol, the growth kinetics correlated well with the Haldane model. The Haldane parameters of psychotropic *P. putida* LY1 were almost within the range reported for other organisms in previous literature. This information on a psychrotrophic organism is of great importance for low temperature bioremediation of contaminated environments.

Razika et al. (2010) used bacterium *Pseudomonas aeruginosa* to eliminate phenol and the benzoic acid. Results obtained show that the microbe is able to degrade phenol as well as benzoic acid. However, it was noted that *Pseudomonas aeruginosa* shows better results in phenol than benzoic acid. However, *Pseudomonas aeroginosa* could not degrade very high concentrations of phenol and benzoic acid completely (> 80 mg/l).

1.5 MECHANISM OF BIODEGRADATION OF PHENOL

1.5.1 AEROBIC BIODEGRADATION

In microbial degradation of phenol under aerobic conditions, the degradation is initiated by oxygenation in which the aromatic ring is initially monohydroxylated by a mono oxygenase phenol hydroxylase at a position ortho to the pre-existing hydroxyl group to form catechol. This is the main intermediate resulting from metabolism of phenol by different microbial strains.

Depending on the type of strain, the catechol then undergoes a ring cleavage that can occur either at the ortho position thus initiating the ortho pathway that leads to the formation of succinyl Co-A and acetyl Co-A or at the meta position thus initiating the meta pathway that leads to the formation of pyruvate and acetaldehyde. Hill and Robinson (1975), Ghadi and Sangodkar, (1995) have described the biodegradation or metabolism of phenol via the meta cleavage pathway, while Gaal and Neujahr (1980) and Paller et al. (1995) described the biodegradation of phenol by *Trichosporon cutaneum* and *Acinetobacter calcoaceticus* respectively via the ortho cleavage pathway.

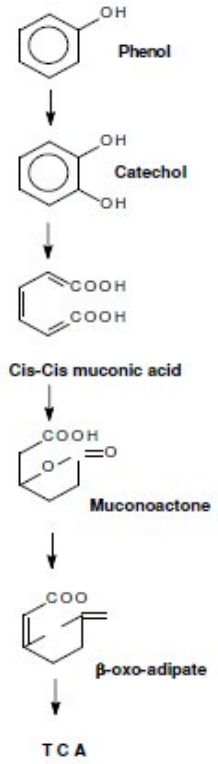

FIGURE 1.2: ORTHOCLEAVAGE PATHWAY OF PHENOL DEGRADATION.

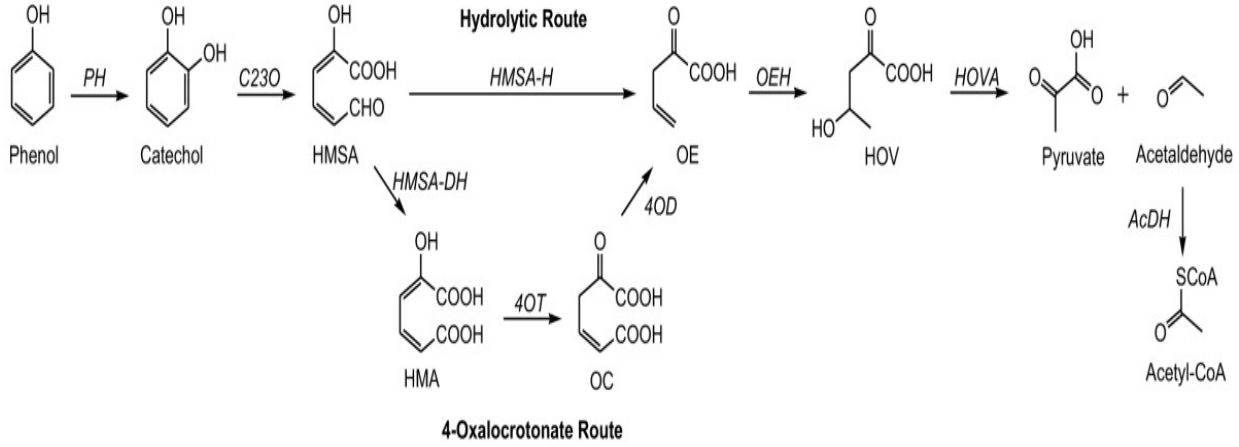

FIGURE 1.3: METACLEAVAGE PATHWAY OF PHENOL DEGRADATION.

1.5.2 ANAEROBIC BIODEGRADATION

The microbial degradation of phenol under anaerobic conditions has been studied in the denitrifying bacterium *Thauera aromatica* (Aresta et al., 1998; Lack and Fuchs, 1992, 1994). The anaerobic degradation is initiated via carboxylation of phenol. The phenol

carboxylation proceeds in two steps. The first step involves the phosphorylation of the phenol by the addition of a phosphate group from an unknown phosphoryl donor catalysed by a phosphorylated enzyme called phenyl phosphate synthase (kinase) to form phenyl phosphate as the first intermadiate (Lack and Fuchs, 1992, 1994). The second step involves the carboxylation of phenyl phosphate catalysed by a Mn^{2+} requiring enzyme, phenyl phosphate carboxylase to form 4-hydroxybenzoate. The synthesis of both the phosphorylating and carboxylating enzymes is strictly regulated. The phenol carboxylating enzyme in *Thauera aromatica* does not belong to any group of the studied carboxylases as it seems to proceed via a phosphorylated free intermediate. It is extremely oxygen sensitive and sensitive to radical trapping agents, it is not dependent on biotin or thiamine diphosphate and differs from most known carboxylases by using carbon dioxide as substrate and a metal as co-catalyst (Breining et al., 2000).

1.6 ENZYMES RESPONSIBLE FOR THE BIODEGRADATION

There are reports on many microorganisms capable of degrading phenol through the action of variety of enzymes. These enzymes may include oxygenases hydroxylases, peroxidases, tyrosinases and oxidases.

1.6.1 OXYGENASES

It is the enzyme that changes the hydrophobic organic compound to more water-soluble and thus it can be broken down by a larger number of other microorganisms. Two major classes of oxygenases are known. They are monooxygenase and dioxygenase. These enzymes participate in the oxidative metabolism of a wide variety of chemicals of pharmaceutical, agricultural and environmental significance. Some of the most widely recognized substrates for this class of enzymes are the aliphatic and aromatic hydrocarbons of both endobiotic and xenobiotic sources.

1.6.2 MONOXYGENASES

This class of enzymes inserts one atom of the oxygen molecule into the substrate, and the other atom of oxygen becomes reduced to water, i.e. two reluctant (substrates) are needed. They are also more complex in action, and can catalyze several different types of O insertion reactions. Since monoxygenases oxidize 2 substrates, they are also called *mixed*

function oxidizes. Also since one of the main substrates becomes hydroxylated, they are also called *hydroxylases*.

The general stoichiometry is as follows:

$$R\text{-}H + NAD(P)H + O_2 \text{ -----> } R\text{-}OH + NAD(P) + H_2O$$

1.6.3 DIOXYGENASES

Dioxygenases incorporate both atoms of the oxygen molecule into the substrates. Dioxygenases are very important in initiating the biodegradation of a variety of chlorinated and nitro-aromatic compounds as well as non-substituted PAHs. Many of these compounds are first degraded to catechol or protocatechuate by oxygenases (both dioxygenases and monooxygenases). The intermediates are metabolized by ring-cleavage type of dioxygenases to either beta-ketoadipate or 2-keto-4-hydroxyvalerate. These intermediates then enter the TCA cycle.

1.6.4 HYDROXYLASE

Phenol hydroxylase catalyses the degradation of phenol via two different pathways initiated either by ortho or meta cleavage. There are many reports on phenol hydroxylase and catechol 2, 3 dioxygenase involved in the biodegradation of phenol (Leonard and Lindley, 1998). Phenol-degrading aerobic bacteria are able to convert phenol into nontoxic intermediates of the tricarboxylic acid cycle via an ortho or meta pathway (Harwood and Parales, 1996). The monooxygenation of the aromatic ring constitutes the first step in the biodegradation of many phenolic compounds. This process is carried out by flavoprotein monooxygenases, which use electrons of NAD(P)H to activate and cleave a molecule of oxygen through the formation of an intermediate flavin hydroperoxide and enable the incorporation of an oxygen atom into the substrate (Moonen et al., 2002). These reactions can be catalyzed by a single polypeptide chain or by multicomponent enzymes (van Berkel et al., 2006). It has been reported as a class of monooxygenases, consisting of a small reductase component that uses NAD(P)H to reduce a flavin that diffuses to a large oxygenase component that catalyzes the hydroxylation of aromatic substrate (van Berkel et al., 2006).

1.7 OPTIMIZATION OF CONDITIONS FOR ENHANCED BIODEGRADATION OF PHENOL

Biodegradation of a substrate by a microorganism depends on a number of factors and it is quite essential to understand how those factors affect the degradation profile of the microbe. Selecting the correct physiological conditions is always a major concern as traditional experiment design would require many experimental runs to achieve satisfactory result.

Jones et al. (1973) have reported that the minimum concentration of phenol was absolutely necessary for the growth of the phenol degrading bacterium NCIB 8250. And the optimum concentration of phenol for maintenance of the organism ranges from 0.24 to 0.28 g/liter.

The enhancement of biodegradation of phenol is accomplished by augmenting the medium with conventional carbon sources. Loh and Wang (1998) have studied on enhancement of the biodegradation potential of the microbe *Pseudomonas putida* ATCC 49451 using sodium glutamate and glucose as the carbon source. They concluded in their study that addition of 1000 ppm glutamate increased the toxicity tolerance of microbes towards phenol significantly. On the other hand, supplementation of glucose caused significant drop in the medium pH from 7.2 to 4.3 resulting in a reduction of degradation rate.

Feitkenhauer et al. (2001) have studied the effect of pH on the phenol biodegradation by the thermophilic strain *Bacillus thermoleovorans* sp A2. They found that highest growth rate was at pH 6.0.

Effect of physiological factors like pH, temperature, concentrations of glucose and ammonium sulfate on the biodegradation potential of *Pseudomonas putida* (ATCC 3180) was studied by Annadurai et al. (2002). They reported that the optimum values for maximum degradation of phenol were pH 7.0, temperature 30^0C, glucose 0.6 g/l and ammonium sulfate 0.6 g/l.

Chakraborty et al. (2010) undertaken an investigation to assess the biodegradation of phenol by native bacterial strain isolated from the effluents of a coke processing plant. The rate of phenol removal by the subjected strain $ESDSPB_2$ was investigated in effect to the various physiological parameters like pH, temperature and glucose concentration of the

medium. The optimal conditions for phenol removal were found to be pH 7, 30^{o}C of incubation temperature and 0.25 % glucose level.

In most of the cases, the desired parameters were either determined based on the experience or as studied in literature. However it does not works for all microbes in all conditions. The optimal growth parameters for even for a particular substrate may vary from microbe to microbe. Thus, several mathematical models based on statistical regression techniques have been constructed to select the proper growth conditions. The most popular model used in case of the parameter optimization is Fractional factorial design. This method is popular because of its simplicity, efficiency and significantly reduces the time. But this very simplicity has led to unreliable results and inadequate conclusions. The number of runs required for full factorial design increases geometrically, which in itself is time consuming and laborious work. Hence the fractional design might not contain the best design point. Moreover, the traditional multi-factorial experimental design is the "change-one-factor-at-a-time" method. Under this method only one factor is varied, while all the other factors are kept fixed at a specific set of conditions.

To overcome these problems, Taguchi and Konishi (1987) advocated the use of orthogonal arrays and Taguchi (1990) have devised a new experiment design that applied signal-to-noise ratio with orthogonal arrays to the robust design of products and processes. In this procedure, the effect of a factor is measured by average results and therefore, the experimental results can be reproducible. Phadke (1989) applied the Taguchi method to design the products and process parameters. This inexpensive and easy to- operate experimental strategy based on Taguchi's parameter design has been adopted to study effect of various parameters and their interactions for the optimal phenol removal by the microorganism. Microbes require optimum conditions for their growth, survival and metabolic activities in the environment. Hence the parameters for taguchi design of experiment will include carbons source, temperature, pH, growth factors and initial substrate concentration *etc.*

1.8 MICROBIAL GROWTH KINETICS FOR SINGLE SUBSTRATE BIODEGRADATION SYSTEMS

Biodegradation of phenol by a pure microbial strain has been extensively studied for more than two decades (Hill and Robinson, 1975; Yang and Humphrey, 1975; Banerjee et

al., 2001; Kumar et al., 2005). Kumaran and Paruchuri (1996) studied the kinetics of biotransformation of phenols using pure microbial cultures and mixture of microbial consortium by fitting the experimental data obtained from a wastewater treatment plant to Monod and Haldane models.

Bandhyopadhyay et al. (1998) have studied the biokinetic parameters of Pseudomonas putida (MTCC 1194). The microbe degraded phenol in the concentration range 100±1000 ppm. The inhibition effects of phenol as substrate have become predominant above the concentration of 500 ppm. Monod model has been used to interpret the free cell data on phenol biodegradation. The kinetic parameters have been estimated upto initial concentration of 500 ppm. The parameter K_S increased drastically with the higher values of initial phenol concentration, but the other parameter μ_{max} decreased slowly with the corresponding increase in the initial phenol concentration, indicating inhibition effect of phenol.

Biodegradation kinetics of benzene, toluene and phenol, both in single and mixed substrate systems using *Pseudomonas putida* F1 was studied by Reardon et al. (2000) where they reported the kinetics of the growth of the culture and developed mathematical models to describe their results. In the tested concentration range, toluene and benzene biodegradation kinetics were well described by the Monod model. Although a small degree of substrate inhibition was noted, the Monod model was able to characterize phenol biodegradation. They reported that Sum Kinetics with Interaction Parameters (SKIP) model provided the best description of the paired substrate results and this model, with parameters determined from one and two substrate systems, provided an excellent prediction of the biodegradation kinetics for the three substrate system.

Goudar et al. (2000) have studied phenol biodegradation in a batch experiment using an acclimatized inoculum with initial phenol concentrations ranging from 0.1–1.3 g/L. They observed that phenol inhibited biodegradation at high concentrations. The experimental results were fitted to the well-known Andrews model and the biokinetic parameters were estimated.

Monterio et al. (2000) have studied the batch phenol biodegradation by *Pseudomonas putida* 548. They varied the initial phenol concentration from 1 to 100 mg/L and observed that the length of the lag phase increases linearly with initial phenol

concentration. The maximum specific growth rate of *Pseudomonas putida* was found to be 0.436 h^{-1}.

Abuhamed et al. (2003) have studied the effect of adaptation of *Pseudomonas putida* F1 ATCC 700007 to the biodegradation of benzene, toluene and phenol. Microorganism growth kinetics was adjusted to the Andrews Kinetics model, which included inhibition terms. In order to investigate the interaction parameters sum kinetics were used. These models provided an excellent prediction of the microorganism growth kinetics and interactions between these substrates.

Kumar et al. (2005) have studied the kinetics of biological degradation of phenol and catechol by *Pseudomonas putida* (MTCC 1194). The microbe was exposed to increasing concentrations of 1000 and 500 mg/l for phenol and catechol, respectively. The microbe degraded 1000 mg/l of phenol completely in 162 hours.

1.9 IMMOBILIZATION OF PHENOL DEGRADERS FOR ENHANCED BIODEGRADATION OF PHENOL

Biodegradation of phenol using pure and mixed cultures of suspended bacteria has been studied in detail. However at higher initial concentration of phenol the growth as well as the degradation activity of the microorganism gets inhibited. Hence a number of strategies have been proposed to overcome the same. Use of microbial immobilization technique is one of them. Cell immobilization is an effective way to maintain continuous substrate degradation with concomitant cell growth for the treatment of toxic materials. Immobilization of microbes for the biodegradation of the xenobiotic substances is the current area of interest in the field of environmental studies. Enhanced stability of the degradation potential, continuous operation and reuse of cells are some of the important advantages of the use of immobilization technique in the biodegradation studies.

Bettmann and Rehm (1984) have reported that on immobilization *Pseudomonas s*p. was able to degrade phenol at an initial concentration of up to 2g/l in less than two days although the free cells did not grow at this concentration. They immobilized the microbe into alginate and polyacrylamide hydrazide (PAAH) and reported that out of the two immobilization supports, the PAAH was more effective.

Ehrhardt and Rehm (1985) have studied phenol biodegradation by immobilized *Pseudomonas* sp. and *Candida* sp on activated carbon. They found that while the immobilized cells tolerated phenol concentration up to 15 g/l and degraded about 90 per cent of phenol within 200 hours, the free cells did not tolerate more than 1.5 g/l phenols. The same authors in another experiment (1989), integrated semi-continuous and continuous degradation of phenol by *Pseudomonas putida* P8 immobilized on activated carbon. The average phenol degradation rate in this process was 360 mg $l^{-1}h^{-1}$. The phenol degradation by free cells of *Pseudomonas putida* p8 in continuous culture was 300 mg $l^{-1}h^{-1}$. But in case of semi continuous and continuous culture, it was up to 2000 mg $l^{-1}h^{-1}$ and 625 mg $l^{-1}h^{-1}$ respectively.

Morsen and Rehm (1990) have reported that the mixed culture of yeast *Cryptococcus elinovii* H1 and *Pseudomonas putida* p8 immobilized on activated carbon and sintered glass degraded phenol at 17 g/L in batch culture whereas in the sintered glass system, it was 4 g/L and during semi continuous degradation of phenol, it was 1g/L both the systems reached constant degradation.

Anselmo et al. (1992) have investigated the phenol biodegradation by free and immobilized cells of *Fusarium flocciferum* in a chemostat at steady-state conditions. The cells were immobilized in polyurethane and it was reported that the immobilized cells were able to completely eliminate the phenol until the initial concentration of phenol is 1g/L but when the concentration of phenol is 1.5g/L the final phenol concentration becomes 248 mg/L.

Chitra et al. (1995) have immobilized *Pseudomonas putida* on rice bran to remove phenol from wastewater. Also, they created mutants to enhance the degradation and reported that the mutant MU 174 degraded 2000 ppm of phenol in 33 hrs.

Bandhyopadhyay et al. (2001) carried out the characterization studies of calcium alginate beads with encapsulated Pseudomonas putida MTCC 1194, used for the biodegradation of phenol. Various techniques were employed to improve the structural stability of the immobilized solid necessary for its use in commercial reactors. Treatment with a hardening agent like glutaraldehyde for different concentrations and treatment times led to variations in structural stability, reusability and the extent of phenol degradation.

Hannaford et al. (1999) have reported the biodegradation of phenol by immobilized pseudomonas putida. They further investigated that the rate of phenol degradation is not affected by alginate concentration in the bead but by the initial concentration of phenol in the medium and the pH of the medium. They found that the best degradation rates are obtained at a phenol concentration of up to 1200 mg mL^{-1} (below which degradation rates were similar). Beyond this, a significant decrease in performance will have to be accepted if it is necessary to treat phenol solutions of typical (1500 mg L^{-1}) to high (4500 mg L^{-1}) concentrations.

Beshay et al. (2002) immobilized *Acinetobacter* sp. strain W-17 on porous sintered glass and it completely degraded 500 mg phenol l^{-1} of phenol in 40 hours, but free cells required 120 hours for degrading the same concentration of phenol. They reported that the immobilized cells can be used 7 times without losing their activity.

Abd-El-Haleem et al. (2003) also reported that the immobilized cells of same *Acinetobacter sp.* W-17 degraded phenol (500 ppm) completely in just 24 hours whereas free cells took as long as 120 hours. Repeated use of immobilized cells revealed that they could be used as much as five times without any loss of activity.

1.10 MOLECULAR APPROACH FOR IDENTIFICATION OF CATABOLIC GENES RESPONSIBLE FOR BIODEGRADATION OF PHENOL

Recent developments in molecular-biology-based techniques have led to rapid and accurate strategies for monitoring, discovery and identification of novel bacteria and their catabolic genes involved in the degradation of xenobiotics. Application of these techniques to bioremediation has also improved our understanding of the composition, phylogeny, and physiology of metabolically active members of the microbial community in the environment. DNA hybridization techniques, using labeled DNA as a specific probe have been used in the past for identification of specific microorganisms in environmental samples. On the other hand, greater sensitivity of detection, without reliance on cultivation, can be obtained using PCR.

Over the past decade, the bacterial degradation of aromatic compounds has frequently been shown to be plasmid encoded (Chakrabarty et al., 1976). Various plasmid

encoded genes like TOL, NAH, SAL and other have also been reported to utilize aromatic compounds in Pseudomonas (Fujii et al., 1997). Amplification of nucleic acids by PCR and reverse transcriptase (RT)/PCR have now been successfully applied in detecting specific genes and their activities in samples containing nucleic acids from diverse communities (Bruce et al., 1992; Selvaratnam et al., 1995). Genes for the degradation of organic pollutants have usually been allocated to plasmid DNAs in bacteria or considered non-mobile when detected in the chromosome.

The meta-cleavage pathway for catechol is one of the major routes for the microbial degradation of aromatic compounds. Shingler et al. (1992) have studied the efficient degradation of phenol by *Pseudomonas* sp. strain CF600 via a plasmid-encoded multicomponent phenol hydroxylase and a subsequent meta-cleavage pathway. They reported that the genes for the entire pathway were found to be clustered and determined the nucleotide sequences of DMP genes which encode the first four biochemical steps of the pathway. They further reported that all the fifteen genes, lie in a single operon structure with intergenic spacing that varies between 0 to 70 nucleotides.

Shingler et al. (1993) have cloned the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway and identified it as a member of the NtrC family of transcriptional activators. The probable role of this domain is the interaction with σ^{54} RNA polymerase, and binding and hydrolysis of ATP to allow formation of open transcriptional complexes.

Kirchner et al. (2003) have described a novel phenol hydroxylase (PheA) that catalyzes the first step in the degradation of phenol in *Bacillus thermoglucosidasius* A7. They reported two-protein system, encoded by the *pheA1* and *pheA2* genes, consists of an oxygenase (PheA1) and a flavin reductase (PheA2) and is optimally active at 55 °C. PheA1 catalyzes the efficient *ortho*-hydroxylation of phenol to catechol when supplemented with PheA2 and FAD/NADH. The hydroxylase activity is strictly FAD-dependent, and neither FMN nor riboflavin can replace FAD in this reaction. PheA2 catalyzes the NADH-dependent reduction of free flavins.

Zhan et al. (2009) have studied the delayed utilization of phenol in the presence of benzoate exhibited by *Acinetobacter Calcoaceticus* PHEA-2. The mphR encoding the transcriptional activator and mphN encoding the largest subunit of multi-component phenol

hydroxylase in the benA mutant were significantly down regulated on adding benzoate to the medium. They suggested that catabolite repression of phenol degradation by benzoate in A. calcoaceticus PHEA-2 is mediated by the inhibition of the activator protein MphR.

Peters et al. (1997) have reported the acquisition of a deliberately introduced phenol degradation operon, *PheBA*, by different indigenous *Pseudomonas* species. Expression of the gene *pheA*, which encodes phenol monooxygenase and is linked to the *pheBA* operon (A. Nurk, L. Kasak, and M. Kivisaar, Gene 102:13–18, 1991), allows pseudomonads to use phenol as a growth substrate. They reported that the *pheA* gene was also represented in the phenol-degrading strains. The *phe* genes were clustered in the same manner in these nine *phe* operons and were connected to the same promoter as in the case of the original *pheBA* operon.

Watanabe et al. (1999) have isolated a total of 41 bacterial strains with different repetitive extragenic palindromic sequence PCR patterns from the activated sludge under different phenol-loading conditions and sequenced the 16S rDNA and *gyrB* fragments of these strains. The *gyrB* phylogenetic analysis divided these strains into two physiologically divergent groups; both of these groups of strains could grow on phenol, analysis showed that population shift occurred following the increase in the phenol-loading rate. This study also demonstrated the usefulness of *gyrB*-targeted fine population analyses in microbial ecology.

Selvaratnam et al. (1995) have showed application of reverse transcriptase PCR for monitoring expression of the catabolic *DmpN* gene in a phenol-degrading sequencing batch reactor. They developed a modified freeze-thaw method in combination with reverse transcriptase PCR for monitoring gene expression in activated sludge. This technique was subsequently utilized to analyze the in situ expression of the catabolic *dmpN* gene in a sequencing batch reactor (SBR) bioaugmented with phenol-degrading *P. putida* ATCC 11172. Greatest *dmpN* expression was observed 15 min after maximum phenol concentration was reached in the reactor and 15 min after the start of aeration. Decreased phenol concentrations in the reactor corresponded to reduced levels of *dmpN* expression.

Futamata et al. (2001) have monitored the specificity of the phenol hydroxylase genes. The sequences of the largest subunit of bacterial multicomponent phenol

hydroxylases (Lmphs) were compared. They reported that Lmphs formed three phylogenetic groups, I, II, and III, corresponding to three previously reported kinetic groups, low-*ks* (the half-saturation constant in haldane's equation for trichloroethylene [TCE]), moderate-*ks*, and high-*ks* groups. Competitive PCR assays were used to quantify Lmphs belonging to each group during the enrichment of phenol/TCE-degrading bacteria from the aquifer soil.

Cafaro et al. (2004) have reported the presence of two different monoxygenases in the microbe Pseudomonas Stutzeri. They found phenol hydroxylase (PH) and toluene/*o*-xylene monooxygenase (ToMO). They earlier reported about the ToMO enzyme and in the current study they expressed and characterized this novel phenol hydroxylase gene. They also mentioned in their study that the phenol hydroxylase has two components, PH P component transfers electrons from NADH to a subcomplex endowed with hydroxylase activity while PH M subunit has been suggested for a regulatory function.

Saa et al. (2010) have identified phenol hydroxylase in Rhodococcus erythropolis UPV-1 in their investigation. They stated that it is a two-component flavin-dependent monooxygenase. The two proteins are encoded by the genes pheA1 and pheA2, which are located very closely in the genome. The sequenced pheA1 gene, was composed of 1,629 bp encoding a protein of 542 amino acids, whereas the pheA2 gene consisted of 570 bp encoding a protein of 189 amino acids. They cloned and characterized both the genes and reported that for hydroxylation of phenol, both the proteins are required in addition to FAD and NADH.

1.11 SCOPE OF THE PRESENT STUDY

Literature study revealed that considerable amount of work has been done on biodegradation of phenol and identification of the genes responsible for encoding the enzymes involved in degradation pathways. However most of the studies were done on the indigenous microbes isolated from the contaminated sites of industrial effluents and waste waters. But often the phenol contamination due to other sources has been overlooked by the scientific community. The idea behind the present study was to explore the microbial community existing in those areas. It is a well known fact that phenol is the major component of most of the disinfectant used in recent time. Hence phenol contamination due

to hospital wastes and sewage is a common problem in the water bodies located in the nearby areas. The present work primarily involves the study on biological degradation of phenol using various bacterial strains. In the present study, soil sample from the dumpsite of hospital wastes is taken for the enrichment study for isolation of soil microorganism present in the contaminated site. The isolated microorganism was characterized by various biochemical and morphological tests for its identification. Several other microbes which are earlier reported for phenol degradation were also subjected for the degradation study and the biokinetic parameters were determined using the obtained data. The enhanced biodegradation of the xenobiote was achieved by immobilizing the microbes in alginate beads. The reference microorganism as well as the isolate were screened against a set of primers that were known to amplify the phenol degrading genes.

1.12 RESEARCH OBJECTIVES

As mentioned in the previous section, most of the research work on the biodegradation of phenol was carried out on the isolation of the microorganism from the contaminated site of the industrial effluents. Moreover acute research work on certain microorganisms like *Pseudomonas resinovorans* have limited our knowledge on the biodegradation potential of the microorganism. Hence based on the available literature, the overall objective of the present study was to evaluate the phenol biodegradation potential of various microbes and the genes present in them which secretes the enzymes responsible for the above.

The specific objectives of the present investigation are the following:

→Isolation, screening and selection of microorganisms, which efficiently degrade phenol.

→Batch shake flask studies on the culture growth and biodegradation of phenol by the isolate.

→Batch shake flask studies on the culture growth and biodegradation of phenol using some reference microbes which are earlier reported to degrade phenol efficiently.

→Estimation of biokinetic parameters involved in the culture growth and phenol biodegradation.

→ Immobilization of phenol degrading strains to improve biodegradation efficiency.

→Isolation of the genes responsible for the utilization of the phenol by the microbe.

1.13 LAYOUT OF THESIS

This thesis comprises of four chapters. The literature that supports the present work is presented in Chapter 1. Details of the materials and methods adopted in the present study are described in Chapter 2. This chapter provides technical information about the quantification of phenol. It also elaborates the details of the enrichment technique adopted for the isolation of the microbial strain, the immobilization study of the subjected microbes for enhancing their phenol degradation capacity. This chapter ends with the description of the DNA isolation and strategies for amplification of catabolic genes responsible for the degradation of phenol. The relevant details for the study have been provided in this chapter. Chapter 3 contains the results and discussions. This chapter starts with a discussion on the identification and characterization of the microorganism. The results obtained from the statistical analysis of the optimization of various parameters like temperature, pH and initial phenol concentration were studied for the isolate. This chapter emphasizes on the biodegradation potential of different other reported microbes which were subjected to different initial concentrations of phenol both in its freely suspended cell form as well as when immobilized on alginate beads. It also elaborates the respiration assay of the isolate at various concentration of phenol to check the dehydrogenase activity of the microbe. Finally the chapter addresses the genetic factor responsible for the biodegradation of phenol in the subjected microbes where it enumerates the phenol hydroxylase genes responsible for phenol biodegradation obtained from the amplification of the microbial DNA using different primers obtained from the literature study. Chapter 4 presents the major conclusions drawn based on the result and discussions of the previous chapter. It also furnishes recommendations for future work in this relevant field. References used in all chapters are compiled at the end of this thesis.

Chapter 2

MATERIALS & METHODS

Chapter 2

Materials & Methods

2.1 CHEMICALS AND REAGENTS

Phenol and chemicals, used in the study, were of analytical grade; glucose and inorganic salts, used in preparing microbial growth media, were of reagent grade. All the chemicals and reagents were procured from Merck®, India and HIMEDIA®, India. Chemicals and kits used for the biochemical characterization of the isolate were obtained from HIMEDIA®, India. The chemicals used for the isolation of bacterial DNA were molecular biology grade and they were procured from HIMEDIA®, India. The primers, reagents and enzymes required for the amplification of the DNA was procured from Banglore Genei, India.

2.2 ISOLATION AND SELECTION OF A PHENOL DEGRADING STRAIN OF BACTERIAL CULTURE BY ENRICHMENT METHOD

2.2.1 SAMPLE COLLECTION

Soil samples were collected from the area contaminated with the sewage discharged from the Government Area Hospital, Sakhigopal, Odisha.

2.2.2 SAMPLING

A soil profile of 1-10 cm under the surface was sampled using a sterile knife after removal of the top layer of the soil up to 1-2 cm. Three subsamples were taken from each point and mixed in a sterile plastic bag. The total mass of the soil sample collected was around 50gram. The soil sample pooled in the container was thoroughly mixed and was stored in ambient temperature for travelling.

2.2.3 SCREENING OF PHENOL TOLERATING STRAIN

5 g of soil collected from disinfectant contaminated area was suspended in 100 ml of minimal medium containing 100 mg/l of phenol as sole source of carbon and incubated in

250 ml flasks at 30°C on an orbital shaking incubator at 200 rpm for a period of 10 days. After incubation period, the soil particles were allowed to settle and 5 ml of particulate free suspension was then used to inoculate a 100 ml minimal medium containing 20 mg phenol (Figure 2.1). Four such transfers were made and every time the enriched population was plated on minimal medium plates containing phenol as sole carbon source. After fourth transfer, a pure isolate capable of growth on phenol was obtained.

2.2.4 PREPARATION OF PHENOL STOCK SOLUTION

The stock solution of phenol was prepared by adding 10g of phenol to double distilled autoclaved water and the volume was made up to 1000ml. The final concentration of the stock solution was 10000 PPM (10000 mg/L) and the stock solution was diluted to the required concentration for its use in the experiments. The stock solution was filter sterilized by passing it through a 0.25µM syringe filter.

2.2.5 GROWTH MEDIUM

Bacteria were cultivated in minimal salt medium (MSM). The MSM composition used in the study is as per Saravanan et al (2008). The composition of the MSM is detailed in *Appendix I*. Phenol (analytical grade) was used as a sole source of carbon and sterilized phenol solution was added directly to MSM at a concentration of 100 PPM. The media was sterilized by autoclaving and the phenol was used after filter sterilization.

FIGURE 2.1: ENRICHMENT SETUP FOR SOIL SAMPLE COLLECTED FROM THE CONTAMINATED SITE.

2.2.6. SCREENING OF PHENOL DEGRADING STRAIN

All the strains isolated by soil enrichment technique were individually inoculated into 10 ml of the mineral salt phenol medium with 250 PPM of phenol. The tubes were incubated on orbital shaker incubator at 150 rpm at room temperature (25-30^{o}C) for 48 hours. The isolates which showed growth in the broth were plated with phenol containing nutrient agar medium to inoculate individually into mineral salt phenol medium with 500 PPM of phenol. The same procedure was repeated with 1000 PPM phenol containing mineral salt medium. The culture which showed growth in mineral salt phenol medium with 1000 PPM of phenol was selected as the phenol degrading strain. The selected culture was purified by repeated streaking and was stored at -20^{o}C as 30% glycerol stock (*Appendix I*). Working cultures were maintained by sub culturing in every two weeks on mineral salt agar slant plates and broth containing phenol.

2.3 IDENTIFICATION OF THE SELECTED BACTERIAL STRAIN

The isolates were identified based on morphological observation and biochemical characterization. The tests involved were cell shape, motility, colony shape, oxygen requirement, catalase, nitrate reduction, indole production, oxidase, gelatin liquefaction, dextrose fermentation, sucrose fermentation, lactose fermentation, citrate utilization, etc (Nagamani et al, 2009). Bergey's manual of determinative of bacteriology was used as a reference to identify the isolates.

2.3.1 MORPHOLOGICAL CHARACTERS

Bacterial strains isolated were examined for colony morphology; cell shape and size of the isolated bacterium were studied using the microscope and Scanning Electron Microscopy.

2.3.2 MOTILITY

Hanging drop slides were prepared from overnight grown cultures and were observed under the microscope.

2.3.3 GRAM STAINING

Gram staining of the isolate was done by smear prepared from overnight viable cultures. The slide was heat fixed and treated with crystal violet for 30s. Rinse gently in a

stream of water allowed to dry blotted, flooded with Grams iodine solution for 1 min, washed with 95% alcohol for 20s and rinsed subsequently with water. After drying it was flooded with saffranin as counter stain for 30s, washed gently with a stream of water, blotted to dryness and observed under immersion microscopes.

2.3.4 BIOCHEMICAL CHARACTERISTICS

2.3.4.1 Catalase Test

Nutrient slants were inoculated with test organisms and were incubated at 30^0C for 24 hours (Blazevic and Ederer, 1975). After incubation, the tubes were flooded with one ml of three per cent hydrogen peroxide and were observed for gas bubbles. The occurrence of gas bubbles was scored positive for catalase.

2.3.4.2 Nitrate Reduction

The isolate was inoculated in the Nitrate broth (*Appendix I*). After incubation, a dropperful of sulfanilic acid and α-naphthylamine (*Appendix I*) was added. If the medium turns red, the result is positive. If the medium does not turn red add a small amount of powdered zinc. If the tube turns red after the addition of the zinc, it means that unreduced nitrate was present. Hence it is a negative result but if the medium does not turn red it is positive result.

2.3.4.3 Indole Test

A loopful of bacteria was inoculated into the tryptone broth. Incubate 48 hours. A few drops of Indole reagent (*Appendix I*) was added to the broth culture. A positive result has a red layer at the top. A negative result has a yellow or brown layer.

2.3.4.4 Oxidase Test

A small amount of organism was transferred from the overnight grown plate on to a sterile swab. A drop of the reagent was placed on the culture on the swab. Positive reactions turn the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions should be ignored.

2.3.4.5. Gelatin Liquefaction

Plates of gelatin agar (*Appendix I*) in triplicates inoculated with cultures were incubated at 30^0C for three days. After incubation, the plates were flooded with 12 per cent $HgCl_2$ solution and allowed to stand for 20 minutes and observed for clear zone around the growth of organism to indicate gelatin liquefaction.

2.3.4.6. Carbohydrate Fermentation

The carbohydrate fermentation slant (*Appendix I*) was inoculated by stabbing the butt down to the bottom of the slant. Result was analyzed after incubation at 37°C for 18 to 24 h. Acid butt, alkaline slant (yellow butt, red slant) means glucose has been fermented but not sucrose or lactose. Acid butt, acid slant (yellow butt, yellow slant) indicates lactose and/or sucrose has been fermented. Alkaline butt, alkaline slant (red butt, red slant) implies that neither glucose nor sucrose or lactose has been fermented.

2.3.4.7. Citrate Utilization

A loopful of bacteria was streaked onto a citrate agar slant (*Appendix I*). It was incubated for 24 to 48 hours with a loose cap. A positive reaction is indicated by a slant with a Prussian blue color. A negative slant will have no growth of bacteria and will remain green.

2.3.4.8. Methyl Red - Voges-Proskauer Test

A loopful of bacteria was inoculated in MRVP broth (*Appendix I*). It was incubated for a time period of 3 to 5 days. After incubation the result was analyzed from the broth. A clear broth indicates that the organism did not grow and cannot be tested. 1 ml of broth was removed and placed into a sterile tube before performing the methyl red test. 3-4 drops of methyl red was added to the original broth. A positive result has a distinct red layer at the top of the broth. A negative result has a yellow layer.

To 1 ml of the culture from the MRVP broth 15 drops of the VP A (Napthol) reagent and 5 drops of the VP B reagent (Potassium Hydroxide) was added. This reaction takes few minutes before a color change. With a positive reaction the medium changes to pink or red. While a negative reaction the broth will not change color or will be copper colored.

2.4 SCANNING ELECTRON MICROSCOPY

Slides were prepared for scanning electron microscopy by centrifuging the overnight grown culture at 10000 rpm for 5 mins. The pellet obtained was washed twice with double distilled water which was followed by fixation in 2.5% glutaraldehyde for 2 hours. To the mixture equal amount of 0.2% of tannic acid was added and dehydrated via successive passages through 30,50,75,90 and 100% ethanol. The Scanning Electron Microscopy (SEM) images were carried out using JEOL (JSM, Japan) Scanning Electron Microscopy attached to an EDX unit, with magnification from 10X up to 400,000X and resolution 3.5 nm.

2.5 ANALYTICAL PROCEDURES

Measurements of phenol were done by UV–vis spectrophotometer [Systronics] both in UV and visible range. For measuring biomass, the samples were centrifuged at approximately 8000 rpm for 20 min. The supernatant was used for phenol determination. The biomass attached to the walls of tubes was resuspended in distilled water and optical density of this suspension was measured against distilled water as reference at 600 nm using UV–vis double beam spectrophotometer. All the transfers were made in UV chamber, and glasswares and medium properly autoclaved. The batch experiments were repeated and the results were found reproducible within acceptable range.

2.5.1. DETERMINATION OF PHENOL IN MEDIUM

Phenol concentrations were determined by a modified colorimetric technique4-aminoantepyrene method as suggested by Yang et al (1975). In this method phenolic material reacts with 4-Amino Antipyrine in the presence of potassium ferricyanide at a pH of 10 to produce a purple-red coloured end product. The detail procedure for determination of phenol has been described *Appendix II*. Twenty-fold dilutions were made to the reagents ammonium hydroxide, potassium ferricyanide, and 4-aminoantipyrine as described in to enable the use of 5-ml phenol samples instead of 100-ml. The calibration curve was prepared (Figure 2.2) and was found linear up to 1 g/l of concentrations with R^2 value of 0.99.

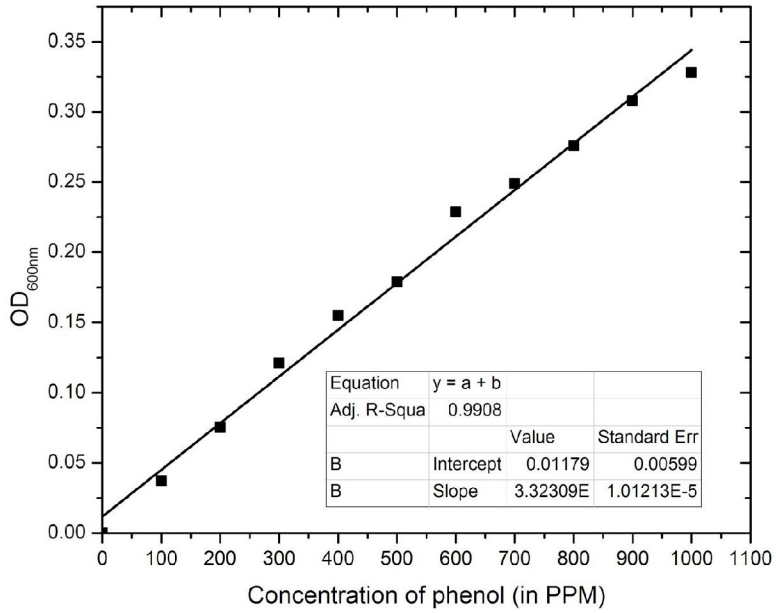

FIGURE 2.2: CALIBRATION PLOT FOR DETERMINATION OF PHENOL BY 4-AMINOANTIPYRENE METHOD.

2.6 OPTIMIZATION OF CONDITIONS FOR ENHANCED BIODEGRADATION OF PHENOL OF THE ISOLATE

2.6.1. DESIGN OF EXPERIMENT

The parameter design of the Taguchi method which includes the following steps as summarized by Trang and Yang (1998) has been carried out in the present study.

1. The quality characteristics and process parameters to be evaluated were identified.
2. The number of levels for the process parameters and possible interactions between the process parameters were determined.
3. The appropriate orthogonal array was selected using the Minitab-14 software and the process parameters were assigned to the orthogonal array.
4. The experiments based on the arrangement of the orthogonal array were carried out.
5. The experimental results were analyzed using the signal-to-noise ratio.

2.6.2. SELECTION OF ORTHOGONAL ARRAY

Many orthogonal arrays are available in other forms such as fractional factorial and Plackett-Burman designs. The selection of parameters and their level was an important step

to design an Orthogonal array (OA). Orthogonal array (OA), L27 each with mixed levels, was chosen (incubation temperature and pH of the media at various initial concentration of phenol). Table 2.1 represents the selected orthogonal array for this study.

2.6.3. S/N RATIO: SIGNAL TO NOISE RATIO

Taguchi approach's based on the analysis of loss function which is used to measure the performance characteristics deviating from the experimental value. The value of the loss functions is further transformed into a signal-to-noise (S/N) ratio. Usually, there are three categories of performance characteristics in the analysis of the S/N ratio.

Minimum /smaller is better is given by

$$S/N_i = -10 \log \left\{ \frac{1}{n} \sum_{j=1}^{n} y_{ij} \right\} \text{ -------- (2.1)}$$

Maximum /Larger is better is given by

$$S/N_i = -10 \log \left\{ \frac{1}{n} \sum_{j=1}^{n} \frac{1}{y_{ij}} \right\} \text{ --------- (2.2)}$$

where, n the number of observations, and y the observed data.

TABLE 2.1: ORTHOGONAL ARRAY FOR THE L_{27} TAGUCHI DESIGN.

Temperature	pH	Concentration
25	6	250
25	6	500
25	6	750
25	7	250
25	7	500
25	7	750
25	8	250
25	8	500
25	8	750

30	6	250
30	6	500
30	6	750
30	7	250
30	7	500
30	7	750
30	8	250
30	8	500
30	8	750
35	6	250
35	6	500
35	6	750
35	7	250
35	7	500
35	7	750
35	8	250
35	8	500
35	8	750

2.7 REFERENCE MICROORGANISMS USED FOR BIODEGRADATION STUDY

The bacterium *Acinetobacter Calcoaceticus* (NCIM 2286), *Pseudomonas Pudita* (NCIM 2650), *Pseudomonas Resinovorans* (NCIM 2599), *Pseudomonas Pictorium* (NCIM 2077), *Pseudomonas Aeruginosa* (NCIM 2074) were procured from National Collection of

Industrial Microbiology (NCIM), National Chemical Laboratory, Pune in lyophilized form. *Arthrobacter sps.* (MTCC 2290), was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbiology (IMTECH), Chandigarh, India, in lyophilized form. These cultures were revived on solid agar Petri dish and in the liquid medium as per the instructions provided by the suppliers. Stock cultures were then obtained by standard spread plate microbial techniques and used for biodegradation study.

2.7.1. PHENOL DEGRADATION STUDIES

All biodegradation experiments were performed in 250 ml Erlenmeyer flask containing 100 ml of MSM containing phenol at concentration ranging from 100 mg/L to 1250 mg/L. Upon incubation of the flasks (Figure 2.3) at 30°C under agitation condition (150 rpm), samples were withdrawn at regular time interval, centrifuged (10,000g for 3 minutes) and analyzed for residual phenol concentration. For each concentration triplicate experiments were performed under the same condition and mean value has been reported. Each experiment was carried out for until the residual concentration of phenol in flask was found to saturate with time and amount of biomass or it is below the detection limit. The reaction mixture containing all components but devoid of bacterial inoculums were used as control.

FIGURE 2.3: BOTH ISOLATED AND REFERENCE MICROBIAL CULTURES DURING BIODEGRADATION STUDY

2.8 MICROBIAL GROWTH KINETICS FOR SINGLE SUBSTRATE BIODEGRADATION SYSTEMS

For any bacteria to grow significantly, the amount of substrate must be sufficiently high as compared to the number of cells to permit sufficient growth of the microbe. If the cell density is high corresponding to the substrate concentration, little or no increase in the cells is possible. Hence it is perceivable that the extent of growth depends on the initial substrate concentration. In order to describe the kinetics of substrate degradation by microbes, several kinetic models such as growth-associated models (logarithmic, logistic and Monod with growth), non-growth associated models (zero order, first order and Monod based) and three-half order models have been reported in the literature (Schmidt *et al*., 1985; Brunner and Focht, 1986). To establish the effect of substrate concentration on growth of microbial culture, specific growth rates of the culture at different substrate concentrations is calculated as per the following relationship:

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \text{---------- (2.3)}$$

where, m is the specific growth rate(h^{-1}), *X* is the biomass concentration (mg/L). Usually, the microbial growth can be represented by a simple Monod equation :

$$\mu = \frac{\mu_m S}{S + K_s} \quad \text{---------- (2.4)}$$

where, *S* is the limiting substrate concentration (mg/L), μ_{max} is the maximum specific growth rate (h^{-1}), *K*s is the half saturation constant (mg/L). On rearranging equation we get,

$$\frac{1}{\mu} = \frac{K_s}{\mu_m}\left(\frac{1}{S}\right) + \frac{1}{\mu_m} \quad \text{---------- (2.5)}$$

The plots were obtained using Origin graphs and the kinetic parameters μ_m , K_s were obtained using the equation (2.4) & (2.5). For each microorganism, different μ_m, K_s value is obtained and they were curved along with respective substrate concentration.

2.9 IMMOBILIZATION OF MICROBIAL STRAINS TO INCREASE THE EFFICIENCY OF PHENOL BIODEGRADATION

2.9.1 PRODUCTION OF INOCULUM FOR USE IN THE PREPARATION OF IMMOBILIZED CELLS

Each microbial strain was inoculated in sterile nutrient broth and was incubated for 24 hours at 30°C, 120 rpm in a 250 ml Erlenmeyer flask. The cells obtained from the media were used for immobilization in the Ca-Alginate beads.

2.9.2. PRODUCTION OF IMMOBILIZED CELLS

Liquid cultures were centrifuged in a 50-ml plastic centrifuge tube (2,500 g) at room temperature for 10 min and the supernatant was discarded. The pellet was resuspended with a previously autoclaved solution of sodium alginate to a final concentration of 4% (w/v) and 10% (v/v) bacterial biomass. The alginate-bacterial mixture was added drop wise with sterile syringe (20 ml) fitted with a wide bore needle (1 mm diameter) from a height of about 20 cm into an autoclaved solution of calcium chloride (3% (w/v), adjusted to pH 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4°C before being harvested by filtration (Figure 2.4).

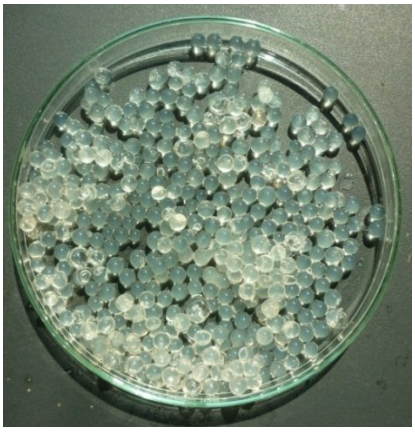

FIGURE 2.4: CELLS IMMOBILIZED IN CALCIUM ALGINATE BEADS

2.9.3. DEGRADATION EXPERIMENTS AND ANALYTICAL METHODS

Phenol degradation experiments were performed in shake flasks with immobilized in the same procedure as followed for the free cells as mentioned in Section 2.5.1. Undegraded phenol left in the media was estimated by the method of Yang and Humphrey (1975) based on rapid condensation with 4-amino-antipyrene as per the protocol in *Appendix II*. Controls

(unimmobilized Ca-alginate beads in MSM phenol medium) demonstrating that phenol was not adsorbed by the immobilizing agent were included with all experiments.

2.10 APPROACH TO TRACE THE GENE RESPONSIBLE FOR BIODEGRADATION

2.10.1. EXTRACTION OF GENOMIC DNA FROM PURE CULTURE

- The bacterial sample was spun till desired amount of pellet was obtained (1ml of culture corresponding to 1 O.D).
- It was washed twice with distill water.
- The pellet was resuspended in 567 µl of TE buffer by repeated pippetting and vortexing.
- In this bacterial suspension 30µl of 10% SDS and 3µl of Proteinase K (stock 20 mg/ml to get a final concentration of 100g/ml Proteinase K in 0.5% SDS) were added and kept for incubation at 37°C for 1hour.
- 80µl CTAB/NaCl solution was added to the above mixture and was mixed thoroughly. It was incubated for 10 minutes at 65ºC.

Phenol Chloroform Treatment:

- About 250µl of Tris saturated phenol and 250µl chloroform was added to the tube after incubation.
- It was thoroughly mixed by inverting the tube carefully.
- The tube was spanned at 12,000 rpm for 10 minute & the upper layer was transferred to a fresh tube.
- Equal volume of chloroform was added & was rocked for 15 minutes.
- The tube was centrifuged at 12,000rpm for 10 minutes & the supernatant was transferred to a fresh tube.
- $1/10^{th}$ volume of 3M-sodium acetate of pH 5.2 & 0.7 volume of Isopropanol was added and incubated at room temp for overnight.
- After incubation it was centrifuged at 14,000rpm for 30 minutes and supernatant was discarded.
- The pellet was washed with 70% ethanol.
- The pellet was air dried completely & dissolved in 50µl double distilled water.

2.10.2 PCR AMPLIFICATION OF THE GENE RESPONSIBLE FOR BIODEGRADATION OF PHENOL

In this study, DNA templates were amplified for detection of gene coding Phenol Hydroxylase or Phenol Monoxygenase enzymes. The study was carried out in presence of primers which were earlier reported for amplification of these genes. PCR (Polymerase Chain Reaction) is a technique widely used in molecular biology, used to amplify specific regions of a DNA strand (the DNA target). It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. After a thorough literature study, several primers which amplify the genes responsible for the biodegradation of phenol were shortlisted for the study. LmPH (largest subunit of multi component phenol hydroxylase) and DmpN (gene coding the N fragment in Pseudomonas Putida-derived methyl phenol operon) are the best example of such type of genes. All the chemicals used in this study were obtained from Banglore Genei, India.

2.10.2.1 PRIMERS USED FOR TRACING THE GENES RESPONSIBLE PHENOL DEGRADATION

The primers used in this study were obtained from literatures available on the microbial phenol degradation. The microbes were screened for the presence of the phenol hydroxylase gene using different primers reported by different available literature. The composition of the reaction mixture (Table 2.2) and the conditions for the reaction were also obtained from the literature.

i) dmpN Primers (Selvaratnam et al., 1995**):**
(5'-ATCACCGACTGGGACAAGTGGGAAGACC-3') and
(5'-TGGTATTCCAGCGGTGAAACGGCGG-3')

ii) Phe1 Primers (Futamata et al., 2001**):**
(5'-GA(G/A)GGCATCAA(A/G)AT(C/T)-3') and
(5'-CAG(C/G)CG(A/G)T(A/T)ACC(G/T)CGCCAGAACC-3')

iii) Phe2 Primers (Futamata et al., 2001**):**
(5'-CC(C/T/G)TTCATGTC(C/G)GG(T/A/C)GC-3') and
(5'- AT(C/T)TG(G/A)TGCAC(C/A)GGCA(G/A)CC -3')

iv) PheU Primers (Futamata et al., 2001**):**
(5'-CCAGG(C/G)(C/G/T)GA(G/A)AA(A/G)GAGA(A/G)GAA(G/A)CT -3') and
(5'-CGG(A/T)A(G/A)CCGCGCCAGAACCA-3')

v) PHP Primers (Cafaro et al., 2004**):**
(5'-CCGGAATTCATATGAGCCAGCTTGTAT-3') and
(5'-CCCAAGCTTAATTCCATTCAAGAATG-3')

vi) Phenol monooxygenase (Baldwin et al.,2003**):**
(5'-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC-3') and
(5'-CGCCAGAACCA(C/T)TT(A/G)TC-3')

2.10.2.2 PCR CONDITION FOR UNIVERSAL DETECTION OF PHENOL DEGRADING BACTERIA (Futamta et al.,2001)

Initial Denaturation: 94°C for 10 mins

Step 1*(5 cycles):*

Denaturation: 94°C for 1 min

Annealing: 58°C for 1 min

Extension: 72°C for 1 min

Step 2*(5 cycles):*

Denaturation: 94°C for 1 min

Annealing: 57°C for 1 min

Extension: 72°C for 1 min

Step 3*(25 cycles):*

Denaturation: 94°C for 1 min

Annealing: 56°C for 1 min

Extension: 72°C for 1 min

Final Extension: 72°C for 10 min

TABLE 2.2: REACTION MIXTURE FOR THE AMPLIFICATION OF THE CATABOLIC GENES (Futamta et al; 2001)

Constituent	Initial Conc.	Volume (in µl)	Final Conc.
10X PCR buffer	200mM Tris HCL 500mM KCL	5	20mM Tris HCL 50mM KCL
$MgCl_2$	25mM	4	2mM
dNTP(Mix)	10mM	1	200µM
Forward Primer Reverse Primer	10pmol/µl 10pmol/µl	0.3 0.3	0.12µM 0.12µM
Template DNA	_____	5	_____
Taq Polymerase	5Unit/ µl	0.4	1Unit
Milli Q Water	_____	34	_____
Total Reaction Volume		**50 µl**	

Chapter 3

RESULT & DISCUSSIONS

Chapter 3

Results & Discussion

One of the most alarming situation in today's world is the generation of a huge amount of waste water contaminated with the toxic organic substances like phenolics from the industrial sector. Phenol is highly water soluble, and its presence in the water imparts a carbolic odor to the receiving water bodies and can have baleful effects on aquatic as well as terrestrial flora and fauna including human beings (ATSDR, 2008). Hence removal of phenol from the discharged sewage and effluent is highly necessary.

Conventional methods for treatment of phenol have their own set of disadvantages hence biological method is the current choice. Reports on possibility of treatment of phenol bearing waste water using microorganisms are also available (Nandish, 2005). Till date, research communities have focused only on the isolation of microorganisms from the sites contaminated with phenol industrial effluents and use them for the treatment of the industrial wastewater. There some other sources like hospital sewage which contains phenol as phenolic derivatives used as disinfectant. Clear Soluble Phenol along with detergent is a widely used as disinfectants in hospitals and in well known disinfectants like stericol, clearsol, etc.

In the present investigation, attempts were made to isolate phenol degrading microorganisms from soil contaminated with hospital sewage after enrichment and to select the most efficient strain for phenol biodegradation. There are certain microorganisms which have been reported by various researchers but detail studies on the biodegradation activity were yet to be done. Hence this study also focuses on the study of phenol biodegradation behavior and identification of the genes responsible for the biodegradation activities of these microorganisms. The present study also aims in the enhancement of the biodegradation behavior of these microbes up on immobilization with suitable support material.

3.1 ISOLATION, IDENTIFICATION AND SELECTION OF AN EFFICIENT PHENOL DEGRADING MICROORGANISMS FROM PHENOL CONTAMINATED SITE

The chances of isolating the microbial strains with high ability to metabolize a particular xenobiotic are brighter from the contaminated soils (Leach et al., 1994). Hence, the soil contaminated with CSP (Clear Soluble Phenol) was chosen as the source of indigenous microorganisms' isolation in this study. Soil samples were collected from dumpsites where hospital wastes as well as the sewage generated from the hospital are been discharged.

Soil sample was enriched in sterile Mineral Salt medium (MSM) using phenol as the sole source of carbon and energy. The sample was further treated with phenol to ensure that only phenol resistant strain would be selected. Up on enrichment with a xenobiotic compound, the natural selection of microorganisms adapted to the presence of a xenobiote have high potential for the biodegradation of the compound (Subramani et al., 1995). Thirty isolates that were able to utilize phenol as sole source of carbon were obtained from the enriched population grown in MSM medium, supplemented with phenol. Enrichment culture technique has earlier been used to isolate several bacteria capable of phenol degradation, which includes *Arthrobacter, Bacillus cereus, Citrobacter, Mircococcus* and *Pseudomonas putida* (Kanekar et al., 1999).

Subsequent exposure to the increasing concentration of the xenobiotic compound to isolate a microorganism which can efficiently degrade it, is a common technique used in the enrichment. Rigo and Alegre (2004) have isolated *Candida parapsilopsis* which degrade $_s$ to 1000 PPM of phenol after screening 22 strains of microorganism. In the present study similar technique has been applied for the isolation of an efficient phenol degrading microorganism. Bacterial strains isolated from phenol enriched soil were evaluated for their ability to grow on phenol by measuring their biomass in terms of optical density (OD). The phenol degradation was measured by estimating the residual phenol remaining in the broth medium. All the thirty colonies isolated by soil enrichment technique were individually subjected to enrichment with MSM. When initial concentration of phenol was 250 ppm, it was interesting to note that all the isolates tolerated the concentrations. Out of these isolates,

only six were selected which yielded more than 80% phenol degradation and they were subjected to higher initial phenol concentration like 500 ppm and 1000 ppm. Of the strains tested, NBM11 showed a higher potential to degrade phenol at both 500 and 1000 ppm which ultimately led to higher biomass production by the strain.

Since NBM11 was more efficient than the other isolated strain, it has been chosen as the subject of the further study. The microorganism was identified based on its morphological and biochemical characteristics. Identification of the strain NBM11 was up to generic level. The morphological characteristic of the strain was studied by plating it on nutrient medium (Figure 3.1). The colony morphology was observed by isolating the single colonies of the microorganism by spread plate technique (Figure 3.2). By comparing the biochemical and physiological characteristics (Table 3.1) of the microbe with those mentioned in Bergey's manual of systematic Bacteriology, the bacterium was identified as *Pseudomonas Sp.*

It is well known fact that *Pseudomonas* is a highly versatile organism with an ability to metabolize even the most complex polymers. It represents the most abundant genus of bacteria in wastewater treatment (Rossello-Mora et al., 1994). The results obtained are in agreement with the findings of (El-Sayed et al., 2003) they have isolated *Pseudomonas aeruginosa* from a coking plant which used phenol as the sole carbon source. Several species of *Pseudomonas as* phenol degraders have been reported by various researchers; *Pseudomonas cepacia* (Ghadi and Sangodhkar, 1995), *Pseudomonas pictorum* (Chitra et al., 1995) and *Pseudomonas putida* (Loh and Wang, 1998; Kanekar et al., 1999).

TABLE 3.1: BIOCHEMICAL CHARACTERISTICS OF ISOLATE *PSEUDOMONAS SP.* STRAIN NBM11.

CHARACTERISTICS	ISOLATE
Gram staining	Gram Negetive
Cell shape	Rod shaped Bacilli
Motility	Motile
Colony shape	Circular, smooth, wet, convex
Oxygen requirement	Aerobic

Catalase	+
Nitrate Reduction	+
Indole Production	-
Oxidase	+
Gelatin Liquefaction	+
Dextrose Fermentation	+
Sucrose Fermentation	+
Lactose Fermentation	-
Citrate Utilization	+
Methyl Red	-
VP	-

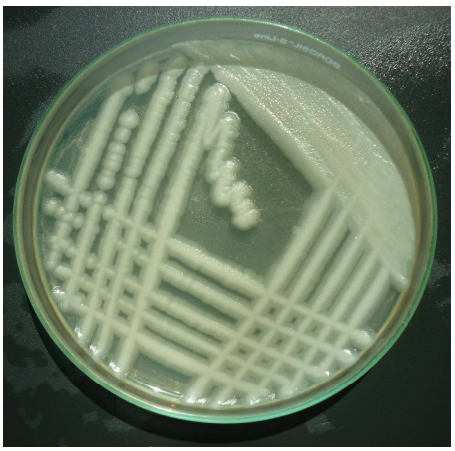

FIGURE 3.1: ISOLATE PSEUDOMONAS NBM11 ON MINERAL MEDIA AGAR PLATE

FIGURE 3.2: ISOLATED COLONIES OF PSEUDOMONAS NBM11 ON MINERAL MEDIA AGAR PLATE

3.2 SEM ANALYSIS OF PSEUDOMONAS SP. NBM11

Scanning Electron Microscopy is evaluated to detect the morphology of the isolated microbial cells. The microorganism was fixed to glass slides with the help of glutaraldehyde fixation method under subsequent drying with increasing concentration of ethanol. The

microbe is fixed in order to maintain the shape of the microorganism under high vacuum condition of the microscopy (Abd-el-haleem et al., 2003). The magnification of the microscopy was 1500X and under high vacuum the images obtained is shown in Figure 3.3. The isolated microorganism is rod shaped bacillus with smooth outer cell walls (Figure 3.3).

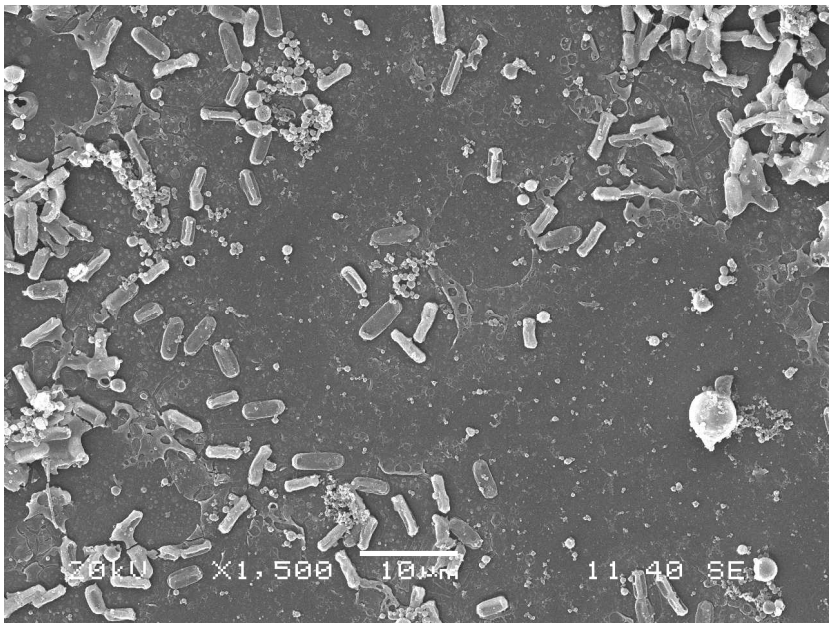

FIGURE 3.3: SEM IMAGE OF THE ISOLATE NBM11.

3.3 OPTIMIZATION OF PHYSIOLOGICAL PARAMETERS OF THE ISOLATE PSEUDOMONAS SP. NBM11 FOR ENHANCED BIODEGRADATION OF PHENOL

Physiological parameters play a vital role in the growth and biodegradation behavior of any microorganism. Microorganism grows within a set range of physiological parameters but maximum growth is achieved only at the optimum conditions of these physiological parameters. Hence determination of the optimum range for the maximum degradation was the aim of the study. Different physiological parameters that usually interfere in the biodegradation activity of a microbe are incubation temperature, pH of the medium, carbon source or source of energy, maximum toxicity of the xenobiote, concentration of micro- and macro nutrient. In the present study, phenol is the source of carbon and energy and hence the physiological parameters to be optimized in this study were incubation temperature and pH of the medium.

The *Pseudomonas sp.* NBM11 was grown in MSM with phenol as the sole source of carbon and energy. The microbe was subjected to varied initial concentration of phenol. Growth in terms of increase in optical density due to increase in biomass was studied under the optimum conditions of temperature and pH at different initial concentration of phenol in this study. The cell growth increased which proves that the strain is able to assimilate the degradation product of phenol. This indicates that higher growth rate of cell will be contributed to higher rate of degradation.

3.3.1 EFFECT OF TEMPERATURE ON GROWTH OF MICROORGANISM

The growth of *Pseudomonas sp.* NBM11 was studied at different incubation temperature from 25°C – 40°C. Figure 3.4 and Figure 3.5 represents the growth curve of the isolate *Pseudomonas sp.* NBM 11 at various temperatures at 250 and 500 PPM of initial concentration of phenol

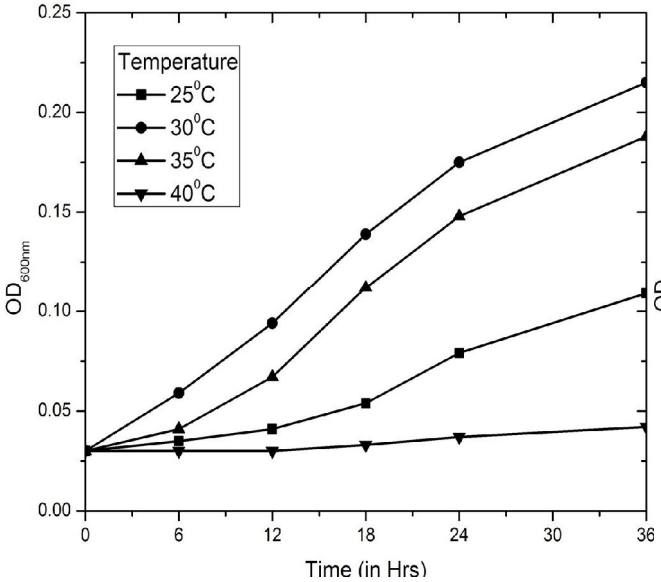

FIGURE 3.4: GROWTH PROFILE OF THE ISOLATE PSEUDOMONAS SP. NBM11 AT VARIOUS TEMPERATURES AT 250 PPM OF INITIAL CONCENTRATION OF PHENOL

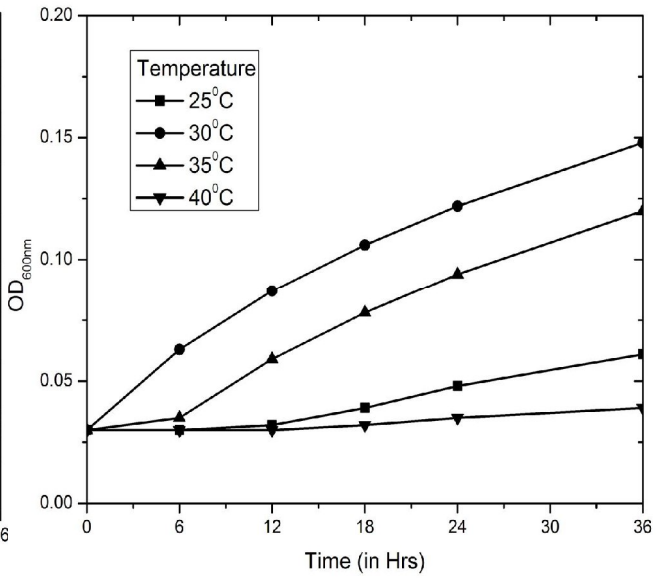

FIGURE 3.5: GROWTH PROFILE OF THE ISOLATE PSEUDOMONAS SP. NBM11 AT VARIOUS TEMPERATURES AT 500 PPM OF INITIAL CONCENTRATION OF PHENOL

. Results from this study indicated that the degradation of phenol was associated with growth of *Pseudomonas sp.* NBM11. High degradation rate was related to high growth rate obtained in cultivation at temperature ranging from 30°C to 35°C. Reduced growth of *Pseudomonas Sp.* NBM11 at temperature (25 & 40°C) caused reduction in the degradation

of phenol. The highest degradation of phenol by the microbe was also obtained in cultivation at 30^{o}C.

3.3.2 EFFECT OF pH ON GROWTH OF MICROORGANISM

The effect of pH on the growth of the microorganism was studied in the range 6-8. The growth curve of the microbe at different pH conditions at 30^{o}C was obtained. Maximum growth of the microorganism was observed at pH 7 at different initial concentration of phenol (Figure 3.6 and 3.7). From the obtained result it was observed that pH of the medium has a significant effect on the growth of the microorganism and ultimately the degradation of the xenobiote.

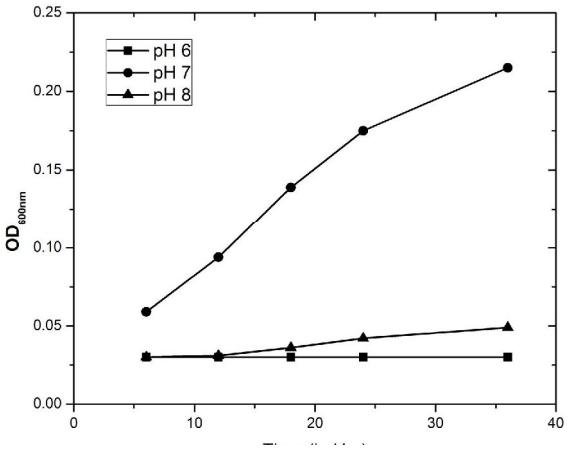

FIGURE 3.6: GROWTH PROFILE OF PSEUDOMONAS SP. NBM11 AT VARIOUS pH CONDITIONS (30^{o}C, 250 PPM OF INITIAL CONCENTRATION OF PHENOL)

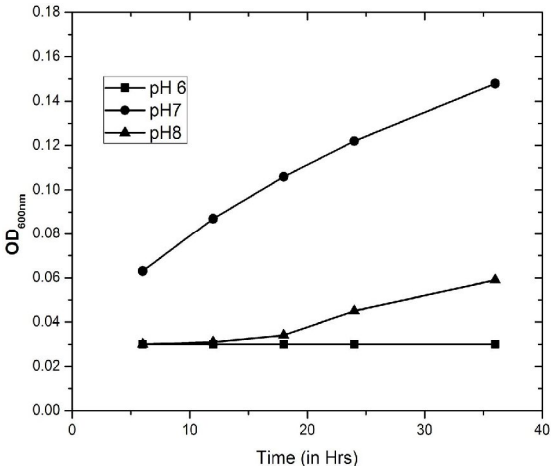

FIGURE 3.7: GROWTH PROFILE OF PSEUDOMONAS SP. NBM11 AT VARIOUS pH CONDITIONS (30^{o}C, 500 PPM OF INITIAL CONCENTRATION OF PHENOL)

3.3.3 EFFECT OF CONCENTRATION ON GROWTH OF MICROORGANISM

The effect of substrate concentration on the growth of the microorganism was studied at various initial concentration of phenol ranging from 250 PPM to 750 PPM at pH 7 and incubation temperature of 30^{o}C. It was observed that with increase in the concentration of the phenol, the substrate confers its toxicity on the growth of microorganism which can be deduced from Figure 3.8. With increase in concentration, the growth of the microorganism slows down.

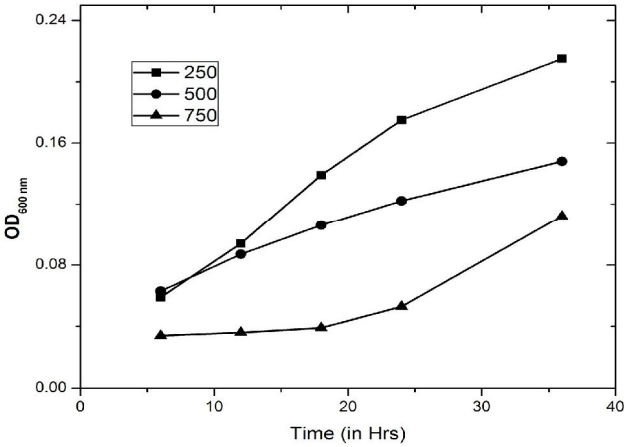

FIGURE3.8: GROWTH PROFILE OF PSEUDOMONAS SP. NBM11 AT VARIOUS INITIAL CONCENTRATION OF PHENOL (30^{O}C, PH 7)

3.4 ANALYSIS OF TAGUCHI DESIGN OF EXPERIMENT

Taguchi techniques are experimental design optimization technique in which we use standard OAs for forming a matrix of experiments that helps the designer to study the influence of multiple controllable factors and the variations in a fast and economic way. Also it allows estimating interaction effects if any and determining their significance (Taguchi et al., 2005). In the present work, experimental work has been designed in a sequence of steps to insure that data is obtained in a way that its analysis will lead immediately to valid statistical inferences. This research methodology is termed as DOE that attempts to extract maximum information with minimum number of experiments.

The data obtained from the experiments conducted as per the Taguchi design to determine the optimal value of each parameter for the microbe. The data were analyzed by Minitab14 software. As mentioned in Section 2.7 two basic factors have been opted for studying their effect on the optimal growth of the microbe. The factors were incubation temperature and pH of the media at different initial concentration of phenol. Table 3.2 shows the result and the corresponding calculated S/N ratio data for phenol removal by the microbe at different combinations of the physiological parameters. To use the S/N ratio for the maximum for the response performances (Table 3.3), S/N calculation was performed and calculated from equation (3.1).

$$S/N = -10*\log \frac{\Sigma[y]^2}{n} \text{ --------- } (3.1)$$

Where n is the number of experiments, N is error.

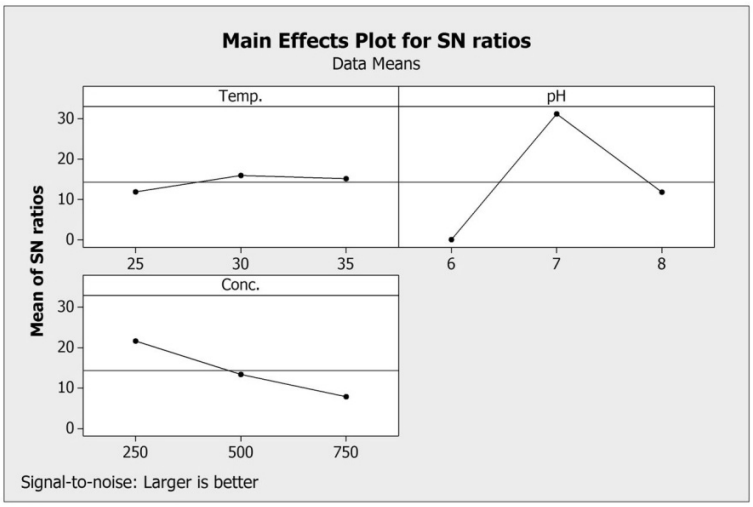

FIGURE 3.9: MAIN EFFECT PLOTS OF PROCESS PARAMETER VS S/N RATIO.

TABLE 3.2: S/N RATIO VALUE FOR DIFFERENT EXPERIMENT PARAMETERS USING TAGUCHI DESIGN.

Temp.	pH	Conc.	% Degradation	S/N ratio
25^0C	6	250	0	2.448279
25^0C	7	250	59.6	35.28093
25^0C	8	250	29.6	27.20155
25^0C	6	500	0	-2.14506
25^0C	7	500	20.8	24.03743
25^0C	8	500	0	-0.30322
25^0C	6	750	0	4.468895
25^0C	7	750	5.8	17.81639
25^0C	8	750	0	-2.24461

30^0C	6	250	0	-1.13114
30^0C	7	250	100.0	40.87806
30^0C	8	250	20.8	26.61434
30^0C	6	500	0	1.016339
30^0C	7	500	62.9	36.37537
30^0C	8	500	4.0	10.6225
30^0C	6	750	0	0.114799
30^0C	7	750	27.2	27.41096
30^0C	8	750	0	1.16562
35^0C	6	250	0	-1.31714
35^0C	7	250	90.8	38.50765
35^0C	8	250	16.4	26.26808
35^0C	6	500	0	1.128718
35^0C	7	500	41.6	34.30335
35^0C	8	500	4.8	10.57463
35^0C	6	750	0	0.188423
35^0C	7	750	21.3	25.30018
35^0C	8	750	0	1.07899

3.4.1 PERCENTAGE DEGRADATION VERSUS TEMPERATURE AND pH OF THE MEDIUM

Larger is better

TABLE 3.3: RESPONSE TABLE FOR SIGNAL TO NOISE RATIOS VALUE FOR DIFFERENT EXPERIMENT PARAMETERS USING TAGUCHI DESIGN.

Level	Temp.	pH
1	11.8401	0.0000
2	15.8963	31.1011
3	15.1148	11.7500
Delta	4.0563	31.1011
Rank	2	1

Table 3.3 expresses the effect of various physiological parameters on phenol degradation. As per the table, the pH of the medium affects the growth of the microbe and phenol biodegradation the most as compared to the incubation temperature. The optimal level of a process parameter is the level with the highest S/N ratio. The degrees of the influences of parameters on phenol degradation is shown in Figure 3.9. Figure 3.10 shows the interaction plot for S/N ratios for different parameters responsible for phenol degradation. The interaction between different parameters was also studied by the contour plot obtained between various parameters. These plots were drawn using the Minitab 14 software and these plots will suggest the best possible range of parameters for the study.

Figure 3.11 represents the contour plot for the phenol biodegradation vs. the pH and Temperature for the growth of the microbe. From the figure it can be inferred that with increasing the temperature for the growth of the microbe the percentage of degradation of phenol decreases. It exemplify that the optimum pH range of the phenol degradation is from 6.5 to 7.5 and the optimal growth temperature is around 30^{o}C.

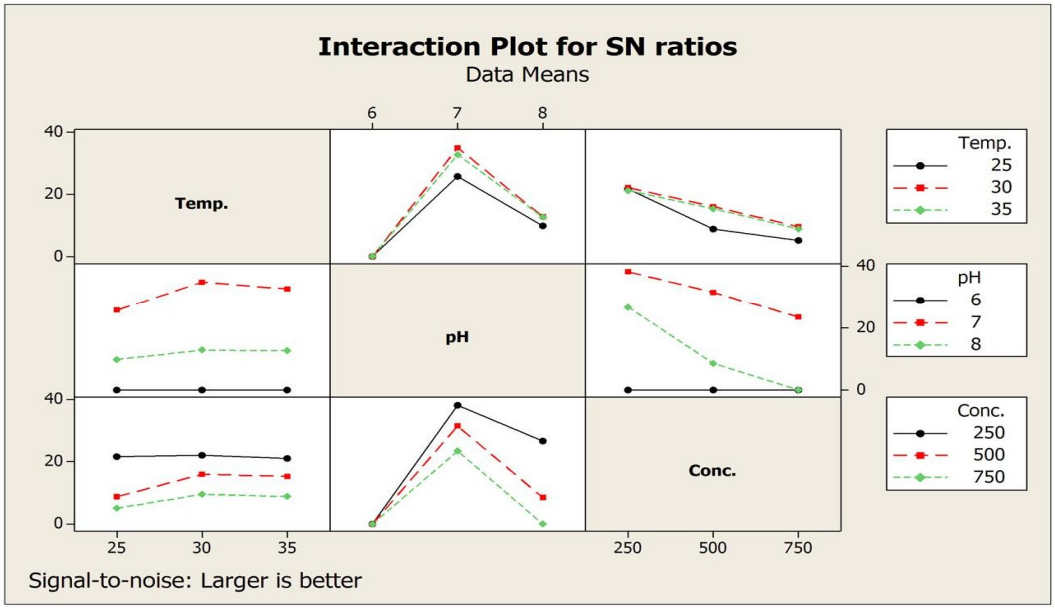

FIGURE 3.10: INTERACTION PLOT FOR SN RATIOS FOR DIFFERENT PARAMETERS RESPONSIBLE FOR PHENOL DEGRADATION.

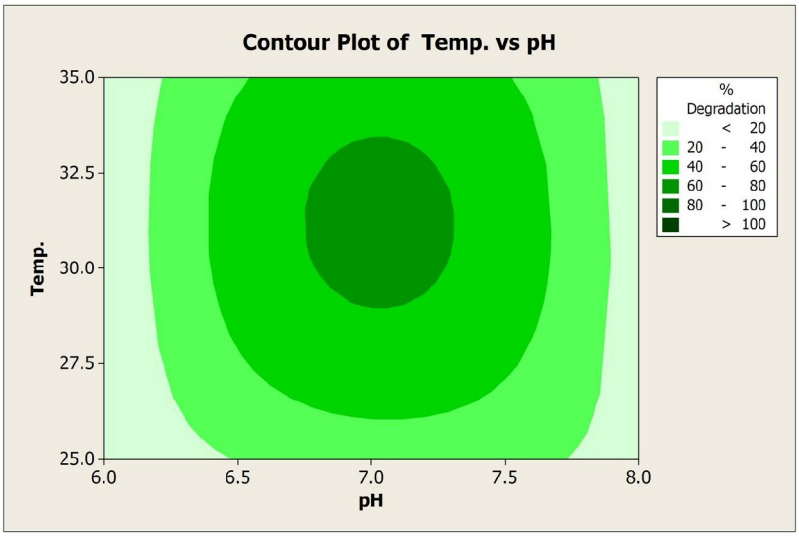

FIGURE 3.11: CONTOUR PLOT BETWEEN % DEGRADATION VS TEMPERATURE, pH

Figure 3.12 indicates the percentage of phenol degradation corresponding to the change in growth temperature with change in the initial concentration of phenol. The Figure delineate that temperature have a less effect on the degradation of the substrate as compared to the concentration of the substrate present initially in the medium. The Figure 3.13 indicates the contour plot between percentage degradation vs pH at various initial

concentration of phenol in the media. From the figure 3.13, it can deduced that the percentage of phenol degradation shown by the isolate is highest when the pH of the medium ranges from 6.5 to 7.5 which is the optimum pH condition for the growth of any mesophillic microorganism. It is observed that with increase in the initial phenol concentration the percentage of phenol degradation decreases. At both end of the extreme pH conditions, the percentage of phenol degradation is below 10%.

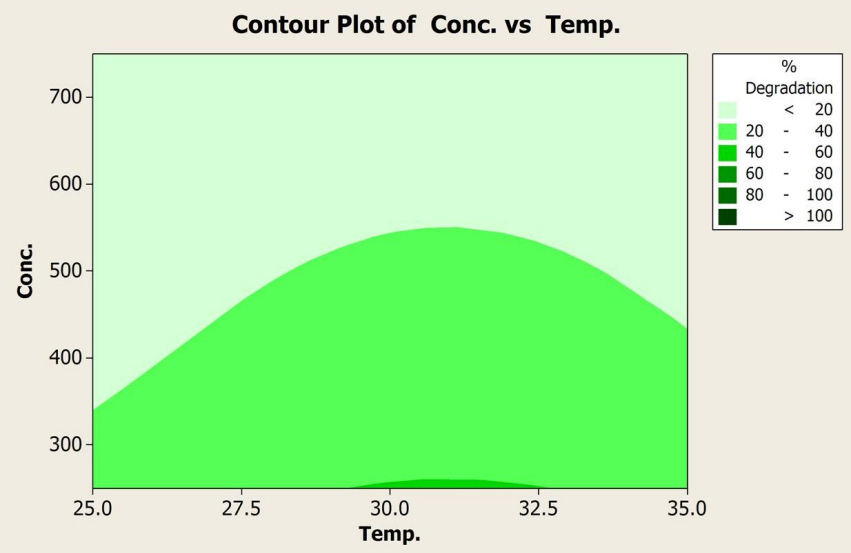

FIGURE 3.12: CONTOUR PLOT BETWEEN PERCENTAGE DEGRADATION VS CONC., TEMP

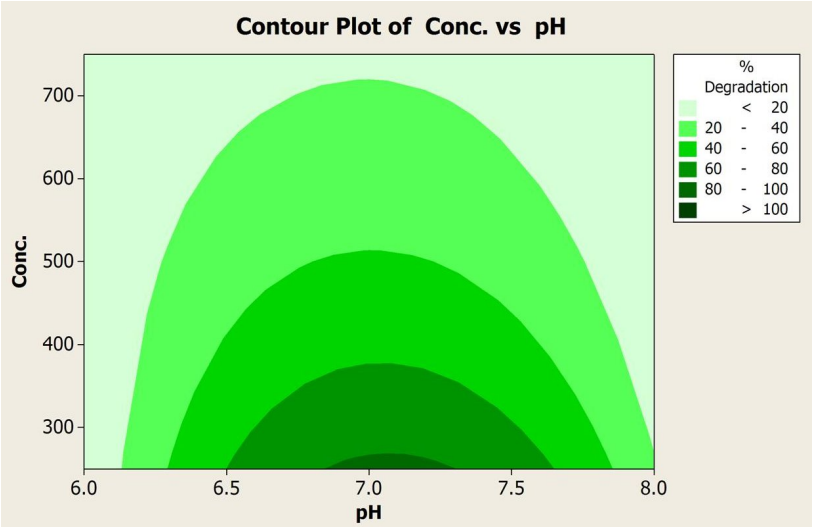

FIGURE 3.13: CONTOUR PLOT BETWEEN PERCENTAGE DEGRADATION VS CONC., PH

3.5 GROWTH AND PHENOL DEGRADATION BEHAVIOR OF PSEUDOMONAS SP. NBM11

The growth and phenol biodegradation study of *Pseudomonas* sp. NBM11 was carried out in MSM broth with phenol as the sole source of carbon and energy. The microbe was subjected to varied initial phenol concentration of 250 - 1250 ppm phenol. The study was carried out in the optimum physiological conditions of incubation temperature of 30^{o}C and pH 7 in an orbital shaker incubator at 200 rpm.

From the enrichment studies carried out (as mentioned in section 3.1), it was known that the microbe is able to tolerate up to a initial concentration of 1000 PPM and is able to degrade the substrate efficiently. Hence the need of this study is to find out the behavior of the organism to the increasing concentration of phenol. Figure 3.14 depicts the growth curve of the microbe at various initial concentration of phenol. The Figure reveals that the microbe can easily tolerate up to 750 PPM of initial concentration of phenol with a gradual increase in the lag phase and decrease in the biomass concentration. With initial concentration of phenol at 1000 PPM there is a stiff increase in lag phase. The increase in the lag phase with increased initial concentration of phenol has been reported by Saravanan et al. (2008) and Bajaj et al. (2009).

Bandyopadhyay et al. (2001) have reported the inhibiting effect of phenol as substrate above the concentration of 600 ppm and 500 ppm respectively. The exponential growth of the microbe corresponding to the rate of degradation clearly indicates that the microbe is able to tolerate such high concentration of phenol and is able to utilize it as its carbon and energy source.

Figure 3.15 represents the degradation behaviour of the strain NBM11 with different initial concentration of phenol. The microbe is able to degrade 250 ppm of initial phenol completely in 36 hrs while it is able to degrade 500 PPM of phenol completely below the detection level in 84 hrs while it degrades 750 PPM of phenol in just 120 hrs and it took 168 hrs to degrade 1000 PPM of initial concentration of phenol completely with a initial lag phase of 48 hrs. At the highest level of phenol (1.2 g/l), growth and phenol degradation was completely inhibited.

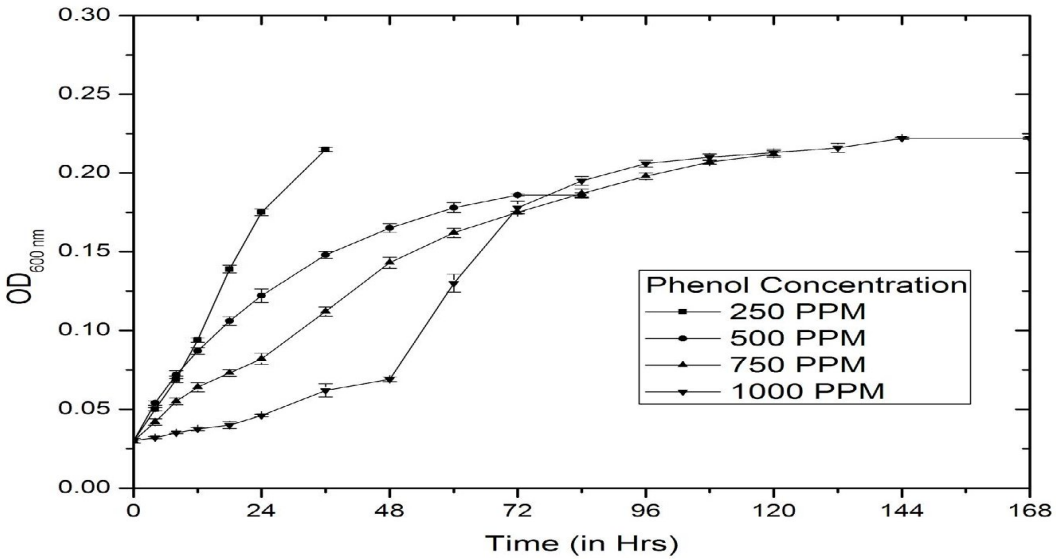

FIGURE 3.14: GROWTH PROFILE OF THE ISOLATE PSEUDOMONAS SP. NBM11 AT VARIOUS INITIAL CONCENTRATION OF PHENOL (30°C, pH 7)

The reason behind this increase in degradation time in case of 750 ppm and 1000 ppm of phenol is the inhibiting effect of phenol at higher substrate concentration above 500 ppm. Saravanan et al. (2008) have reported that microorganisms did not show any inhibitory effect and almost no lag phase was observed during its growth between 100 mg/l and 500 mg/l. The most probable reason behind the extended lag phase is due to the increasing toxicity of phenol with increase in its concentration.

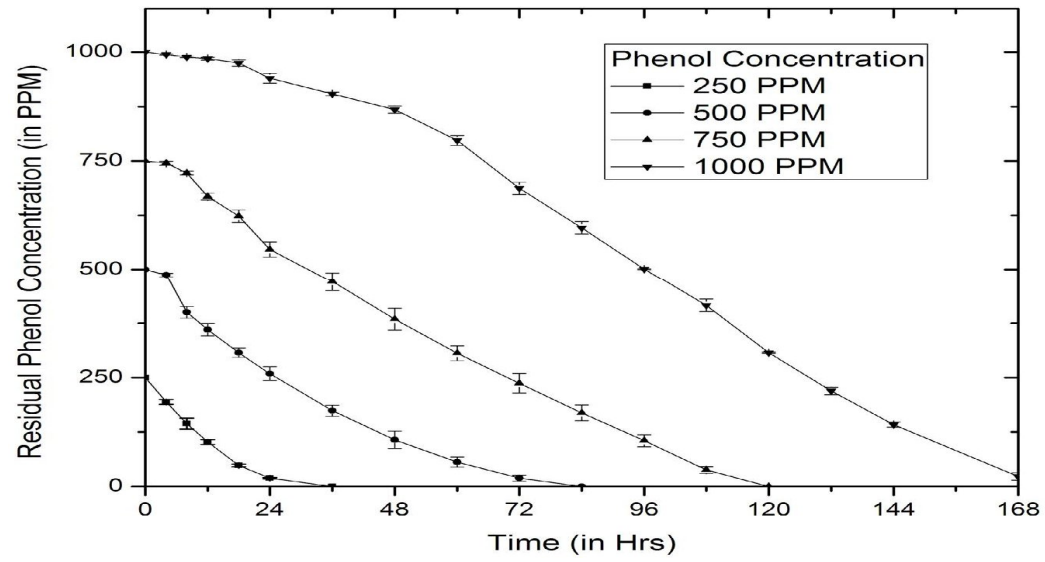

FIGURE 3.15: DEGRADATION PROFILE OF THE ISOLATE PSEUDOMONAS SP. NBM11 AT VARIOUS INITIAL CONCENTRATION OF PHENOL(30°C, pH 7)

The isolated *Pseudomonas* species has been found to degrade high concentration of phenol. To check the maximum limit of tolerance of the microbe towards the substrate, the microorganism was subjected to different initial concentrations of phenol from 1000 – 1250 PPM with an interval of 50 PPM. The microorganism is not able to degrade the substrate completely when initial concentration of the phenol is above 1000 PPM. Increasing substrate concentrations enhanced the growth of isolate up to 500 PPM of phenol, but further increase in the concentration retarded the cell growth of the same isolate.

Figure 3.16 depicts the degradation potential of the microbe *Pseudomonas Sp.* NBM11 at higher concentration of phenol. It was observed that with increase in the concentration of the substrate the degradation potential of the microbe decreases and thus it is completely inhibited at a substrate concentration of 1150 PPM of phenol. The microbe was able to degrade the substrate completely till the initial concentration of phenol in the medium was 1000 ppm. But with increase in the concentration of phenol above 1000 ppm the degradation of the substrate decreases and increase in the lag phase depicted by the microorganism.

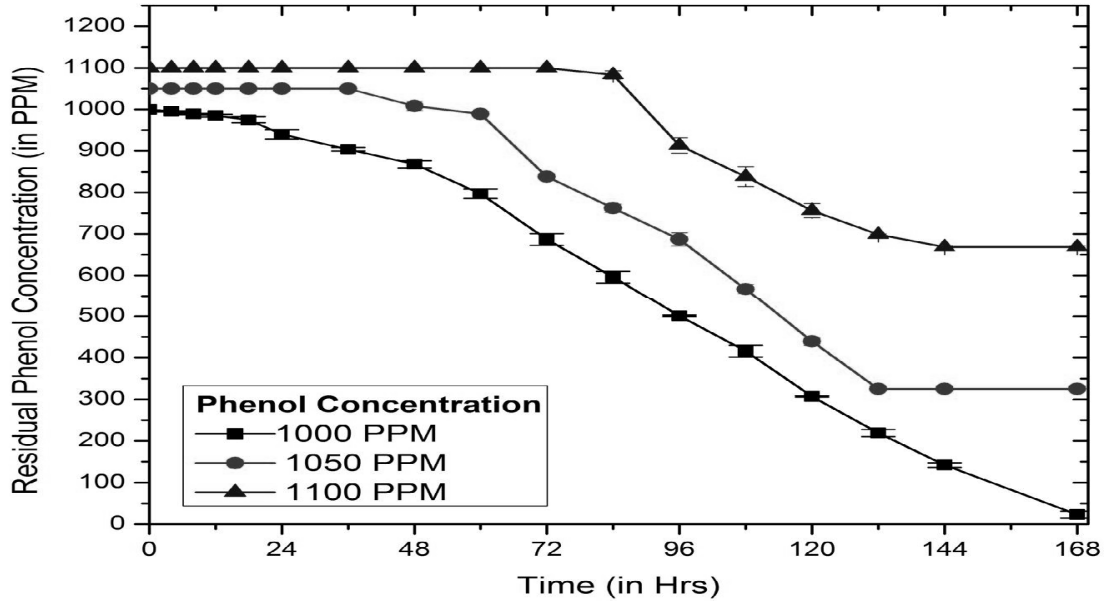

FIGURE 3.16: DEGRADATION PROFILE OF THE ISOLATE PSEUDOMONAS SP. NBM11 AT HIGHER INITIAL CONCENTRATION OF PHENOL (30^{o}C, pH 7)

Several reports have shown that microbial growth and concomitant biodegradation of phenol are hindered by the toxicity exerted by high concentration of substrate itself (Kargi

and Eker, 2005). High concentrations of phenol are usually inhibitory to the growth of microorganisms. Phenolic compounds have the abilities to partition into membranes, disrupted membrane functions and caused cell death. Whiteney and coworkers (2001) found that the isolates of *P. fluorescens* and *P. putida* were effectively insensitive to phenol and showed virtually no decrease in the respiratory response over the phenol concentration up to 400 mg/L.

3.6 STUDY OF PHENOL BIODEGRADATION BEHAVIOUR OF REFERENCE MICROBES

Several microorganisms were discovered to have excellent capability of phenol degradation. Identification of these bacteria showed the dominance of genus *Pseudomonas*, especially *Pseudomonas putida* mainly because of its spread distribution in soils. *Pseudomonas putida* have been studied the most for its phenol biodegradation potential. Bandopadhyay et al. (1998), Kumar et al. (2005), Li et al. (2010), Kargi & Eker (2005), Movahedyan et al. (2009) reported the phenol degradation capacity of the *Pseudomonas putida* in their study.

Anli et al. (2007), Schirmer et al. (1997), Liu et al. (2009) and Zhan et al. (2009) have reported the phenol biodegradation by *Acinetobacter calcoaceticus*. They had shown the presence of mphN gene which encodes the largest multicomponent of phenol hydroxylase in *Acinetobacter calcoaceticus* and hence investigation till the genetic level is completed in this microorganism also. Biodegradation of phenol by *Pseudomonas pictorum* was investigated by Chitra et al. (1996) and Annadurai et al. (2000) in their study. Zheng et al. (2010), Agarry et al. (2008), Hank et al. (2010) in their study have reported the biodegradation activity of *Pseudomonas aeruginosa*. Yang and Lee (2006) in their investigation have reported the biodegradation capability of the microbe *Pseudomonas resinovorans* but till date no further work has been done on the microbe. *Arthrobacter sps*. has been reported several times for its biodegradation potential of various poly aromatic compounds. Kar et al. (1997), Swaminathan (2005) have reported about the biodegradation potential of the microbe. But the microbe's maximum degradation potential and the gene which is responsible for its ability to degrade the xenobiote is yet to be studied.

Even if phenol biodegradation potential of the microbes *Pseudomonas pictorum* and *Pseudomonas aeruginosa* has been reported but not much work has been done on the gene responsible for the biodegradation potential of the microbe which is yet to be determined. Similarly microbe like *Pseudomonas resinovorans* is new to this field and extensive research on the biodegradation potential of the microbe has not been explored well. The degradation potential as well as genetic aspect of the microbe is yet to be studied and hence it has been included in the present study.

After a thorough literature study, it was concluded that microbes like *Pseudomonas pictorum, Pseudomonas aeruginosa, Pseudomonas resinovorans* and *Arthrobacter sps*, were the subject of study for the investigation of their phenol degradation potential, their degradation kinetics, and the gene responsible for the biodegradation activity precisely at the same initial concentrations of phenol in order to draw a consensus of which microbe degrades the substrate faster and their tolerance in the similar physiological conditions. *Pseudomonas putida* and *Acinetobacter calcoaceticus* has considered to serve as the standard microorganisms in monitoring whether the procedures adapted in the study and outcome of the experiments were correct or not.

3.6.1 PHENOL BIODEGRADATION BY ACINETOBACTER CALCOACETICUS

The strain *Acinetobacter calcoaceticus* was subjected to different initial concentration of phenol from 250- 1000 ppm with mineral salt medium. The phenol concentration was estimated at regular interval of time using the standard 4-aminoantipyrene method recommended by Yang et al. (1975). Figure 3.17 represents the degradation characteristics of the *Acinetobacter calcoaceticus* at different initial concentration of phenol. From the figure is observed that the microbe is able to degrade 250 PPM of phenol within 48 hours while it takes 72 hours for the complete degradation of 500 PPM of initial concentration of phenol. The microbe has been claimed for its capability to reduce up to 1000 PPM of phenol below the detection level (Anli et al., 2007) and the degradation curve is in agreement with the literature. The microbe is able to degrade 1000 PPM of phenol in just 144 hrs. Similar results has been reported by Liu et al. (2009) where they have subjected the microbe to 800 ppm of phenol and the microbe was able to degrade it up to 99%.

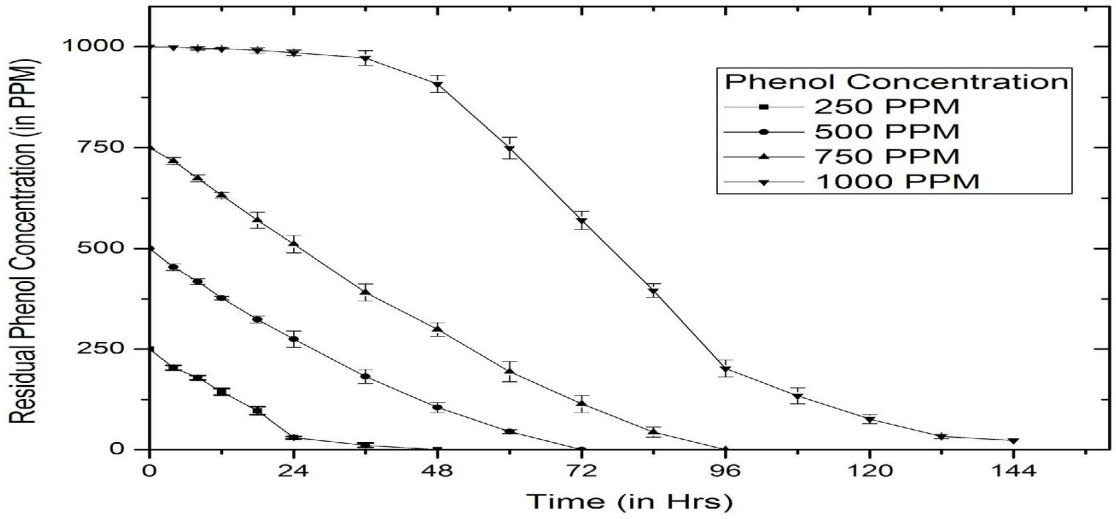

FIGURE 3.17: DEGRADATION CURVE OF *ACINETOBACTER CALCOACETICUS* AT DIFFERENT INITIAL CONCENTRATION OF PHENOL (30°C, pH 7)

3.6.2 PHENOL BIODEGRADATION BY PSEUDOMONAS PUTIDA

Figure 3.18 shows the biodegradation potential of the strain Pseudomonas putida. The microbe is able to tolerate up to 1000 PPM of phenol and can completely degrade it in 144 hours. The microbe is able to degrade 250 PPM of phenol in just 24 hours while it is able to degrade 500 PPM of phenol in 84 hrs. At each of the initial concentrations there was a period of exponential growth when the substrate was being consumed at faster rate. With increase in the concentration of the substrate, lag phase exhibited by the microorganism increases. The concentration of the phenol above 1000 PPM inhibits the growth of the microbe indicating that the increased concentration of the substrate is toxic for the microbe and the microbe is not able to utilize it for its metabolism. Similar results were reported by Kumar et al. (2005) where the strain *Pseudomonas putida* degraded 1000 PPM of phenol in 162 hrs. Li et al. (2010) reported that the *Pseudomonas putida* isolated in their study degrades up to 800 ppm of initial concentration of phenol and is unable to tolerate the phenol concentration above it. The difference in the results may be attributed to the difference in the growth medium and incubation temperature of the experiments.

Watanabe et al. (1998) categorized *Pseudomonas putida* in the high K_s group and stated that the microbe could tolerate and degrade phenol up to 1000 PPM of phenol. Results of various studies show that the higher is the concentration of the compound, the more time it takes to be consumed fully (Bandopadhyay et al., 1998).

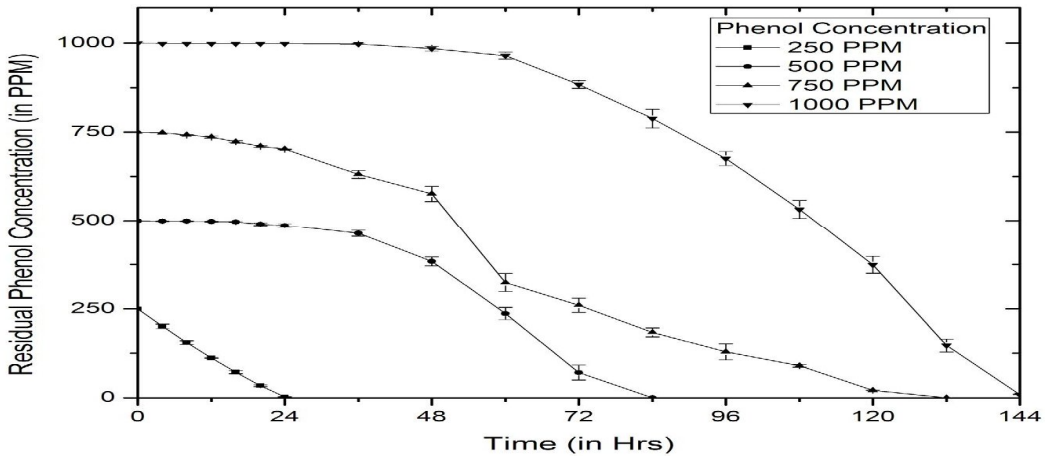

FIGURE 3.18: DEGRADATION CURVE OF *PSEUDOMONAS PUTIDA* AT DIFFERENT INITIAL CONCENTRATION OF PHENOL (30^{0}C, pH 7)

In the present study a t high concentrations, the lag phases has been observed, even though the well-acclimatized inoculum was used in the experiments. The lag-phase of as long as one week had been reported during degradation of phenol at initial concentrations of 700 mg/l using well-acclimatized *P. putida*. Hill and Robinson (1975) have reported that not only inhibitory effect of the substrate but also the size of the inoculum might affect the duration of the lag phase. Therefore, to avoid lag-phase a large amount of inoculum should be used.

3.6.3 PHENOL BIODEGRADATION BY PSEUDOMONAS AERUGINOSA

Figure 3.19 represents the degradation profile of the microbe *Pseudomonas aeruginosa* at various concentration of phenol used as the sole source of carbon and energy. The microbe is able to degrade up to 750 PPM of phenol in the media but shows an extended lag phase with increase in the concentration of phenol. Above 750 PPM of phenol, the growth of the microbe was inhibited and the microbe was no longer able to degrade phenol. It can be concluded that the maximum tolerance of the microbe is 750 PPM. The microbe is able to degrade up to 48% of the initial phenol when the concentration of the substrate is 750 PPM. The microbe degrades 72% of phenol from the media in 144 hrs when the initial concentration of the media is 500 PPM. Zheng et al. (2010) have reported that the microbial strain *Pseudomonas aeruginosa* HSD38 is able to degrade up to 500 PPM of phenol below the detection level but unable to tolerate more than 700 PPM of initial concentration of phenol.

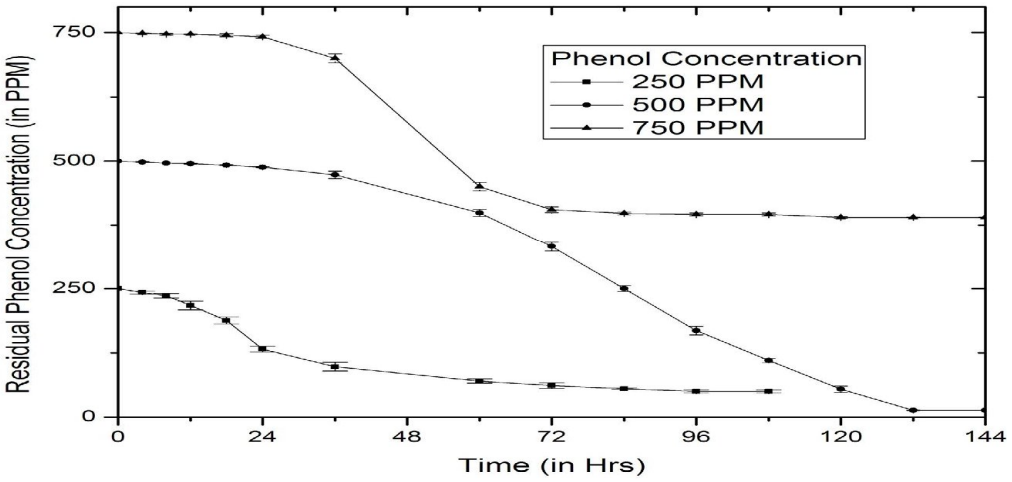

FIGURE 3.19: DEGRADATION PROFILE OF PSEUDOMONAS AERUGINOSA AT DIFFERENT INITIAL CONCENTRATION OF PHENOL (30°C, pH 7).

3.6.4 PHENOL BIODEGRADATION BY PSEUDOMONAS PICTORUM

Figure 3.20 shows the degradation pattern of the microbe *Pseudomonas pictorum*. It is notable to see that the microbe is also not able to degrade the substrate completely but has the potential to withstand such high concentration of phenol as 1000 PPM. The microbe degrades only 69 % of 500 PPM of phenol and on increasing the concentration of the phenol its capacity to degrade the substrate decreases but the microbe is able to grow even in the environment. From the figure 3.20 it can be seen that the microbe exhibit a lag phase at higher concentration of phenol thus implicating that the substrate at higher concentration is lethal to the microbe.

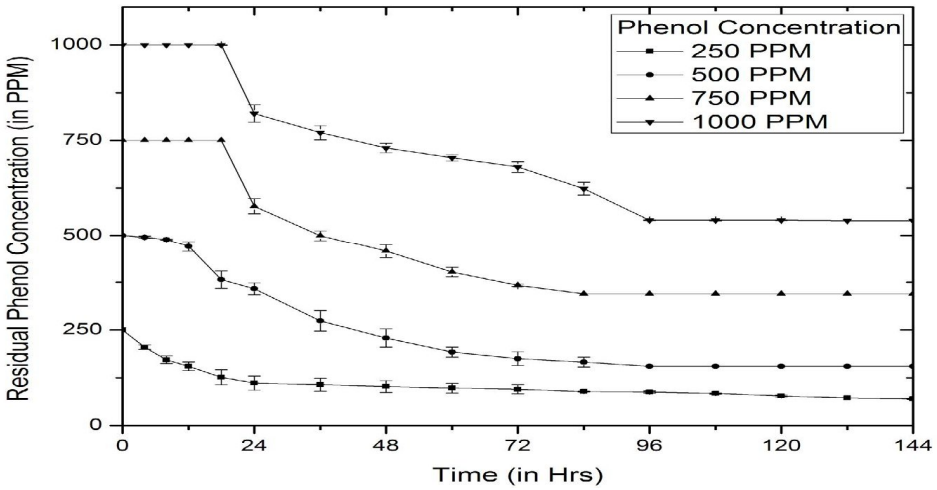

FIGURE 3.20: DEGRADATION BEHAVIOUR OF PSEUDOMONAS PICTORUM AT VARIOUS INITIAL CONCENTRATION OF PHENOL (30°C, pH 7).

3.6.5 PHENOL BIODEGRADATION BY PSEUDOMONAS RESINOVORANS

Figure 3.21 represents the degradation profile of the microbe *Pseudomonas resinovorans*. Contradicting the earlier theory available on its biodegradation potential the microbe is able to tolerate up to 750 PPM of phenol with an extended lag phase but a linear degradation curve. The microbe shows an efficient degradation potential up to 500 PPM of phenol where it completely degrades the substrate in 96 hrs.

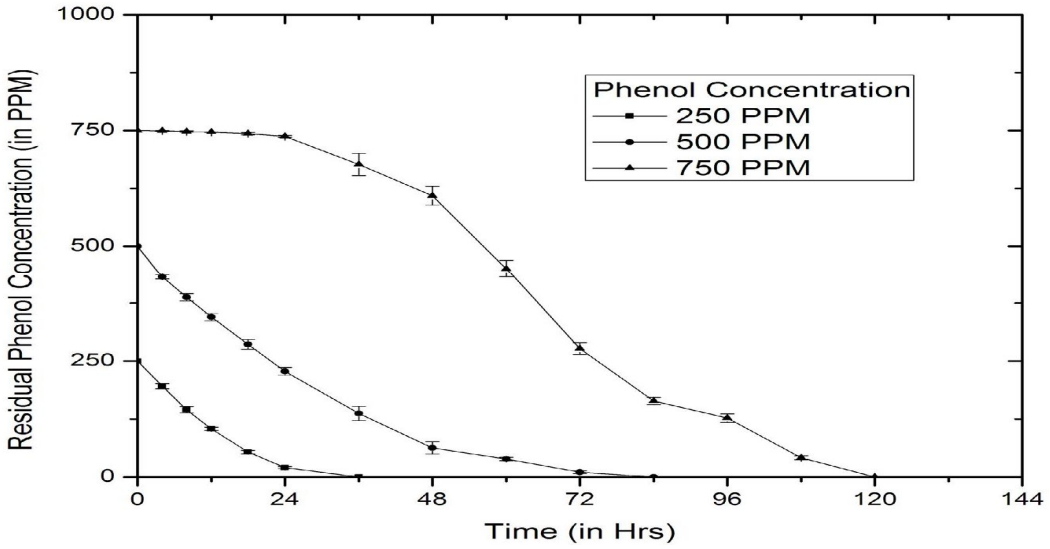

FIGURE 3.21: DEGRADATION BEHAVIOUR OF PSEUDOMONAS RESINOVORANS AT VARIOUS INITIAL CONCENTRATION OF PHENOL (30^{o}C, pH 7).

Yang and Lee (2006) have reported that the microbial strain *Pseudomonas resinovorans* isolated and studied in their investigation was unable to tolerate an initial concentration of phenol of more than 600 PPM. But in the current investigation, the microbial strain not only showed tolerance for 750 PPM of phenol but also completely degraded the substrate within 120 hours. But on increasing the concentration of the phenol above 750 PPM inhibits the growth of the microorganism completely even on incubating it for a prolonged time period.

3.6.6 PHENOL BIODEGRADATION BY ARTHROBACTER SP.

Figure 3.22 depicts the phenol biodegradation behaviour of the Arthrobacter sps at different initial concentration of phenol. The microbe is able to degrade up to 96 % and 52 % of the initial phenol when the initial concentration of the substrate in the media is 500 PPM and 1000 PPM respectively. Even if the microbe is not able to utilize the substrate

completely, it is able to tolerate such high concentration of phenol which makes it a promising candidate in the field of phenol biodegradation.

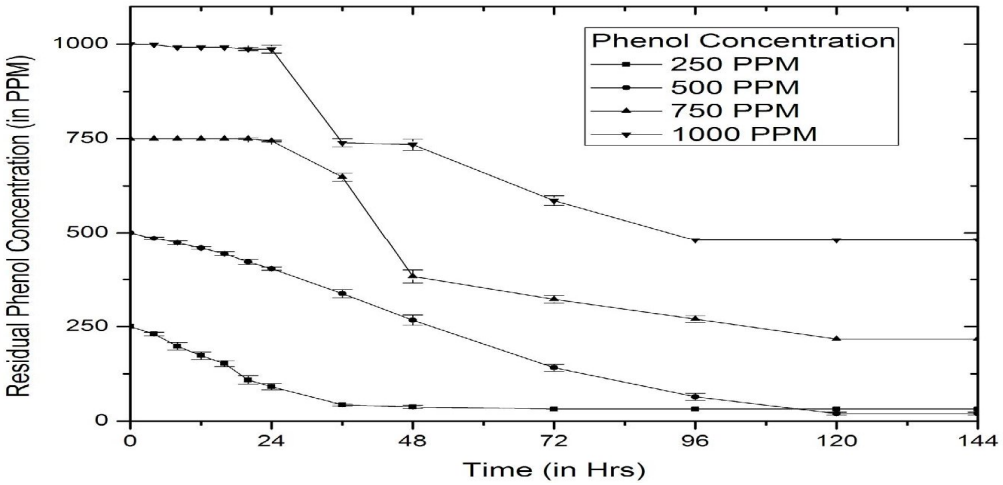

FIGURE 3.22: DEGRADATION BEHAVIOUR OF ARTHROBACTER SPS. AT VARIOUS INITIAL CONCENTRATION OF PHENOL

The obtained result has been substantiated by the results reported by Margesin et al. (2004). They reported that the microbe is able to tolerate up to 10mM of phenol. Chakraborty et al. (2010) and Kariger et al. (2005) reported that the microbial strain *Arthrobacter citreus* is able to mineralize up to 5mM of initial concentration of phenol completely and can tolerate up to 22mM of initial concentration of phenol.

3.7 COMPARISON OF THE BIODEGRADATION POTENTIAL OF PSEUDOMONAS SP. NBM11 WITH THE REFERENCE MICROBES

The biodegradation potential of the microbe *Pseudomonas sp.* NBM11 as well as other reference microbes was studied individually at different initial concentration of phenol. Each microbe has a different profile of degradation of phenol and the tolerance limit. The individual study of various microbes has been carried out to compare the phenol degrading capacity of the isolate with the other reference microbes. All the microbes were grown under same physiological conditions and initial concentration of phenol. The microbes were grown on mineral salt media with four different concentration of phenol (250, 500, 750 & 1000 ppm). Since the isolated microbe *Pseudomonas sp. strain NBM11* is able to completely degrade up to 1000 PPM of initial concentration of phenol and with a substrate

concentration above this its growth gets inhibited, throughout this study the maximum degradation potential has been set to 1000 ppm for each and every investigation.

3.7.1 AT 250 PPM OF INITIAL CONCENTRATION OF PHENOL

When the microbes were subjected to initial concentration of 250 ppm of phenol, the degradation behavior observed is shown in figure 3.23. All the microbes were able to degrade the substrate completely but time taken by each of the microbe depends on the degradation potential of the microbe. *Pseudomonas sp.*NBM11 is able to degrade the substrate completely within 36 hours while *Pseudomonas putida* and *Acinetobacter calcoaceticus* degraded the substrate completely within 48 hrs each with different degradation rate. Even *Pseudomonas resinovorans* is also able to degrade the substrate completely within 48 hours. Thus *Pseudomonas sp.* NBM11 has the best degradation ability among all other microbes when the initial concentration of phenol is 250 ppm.

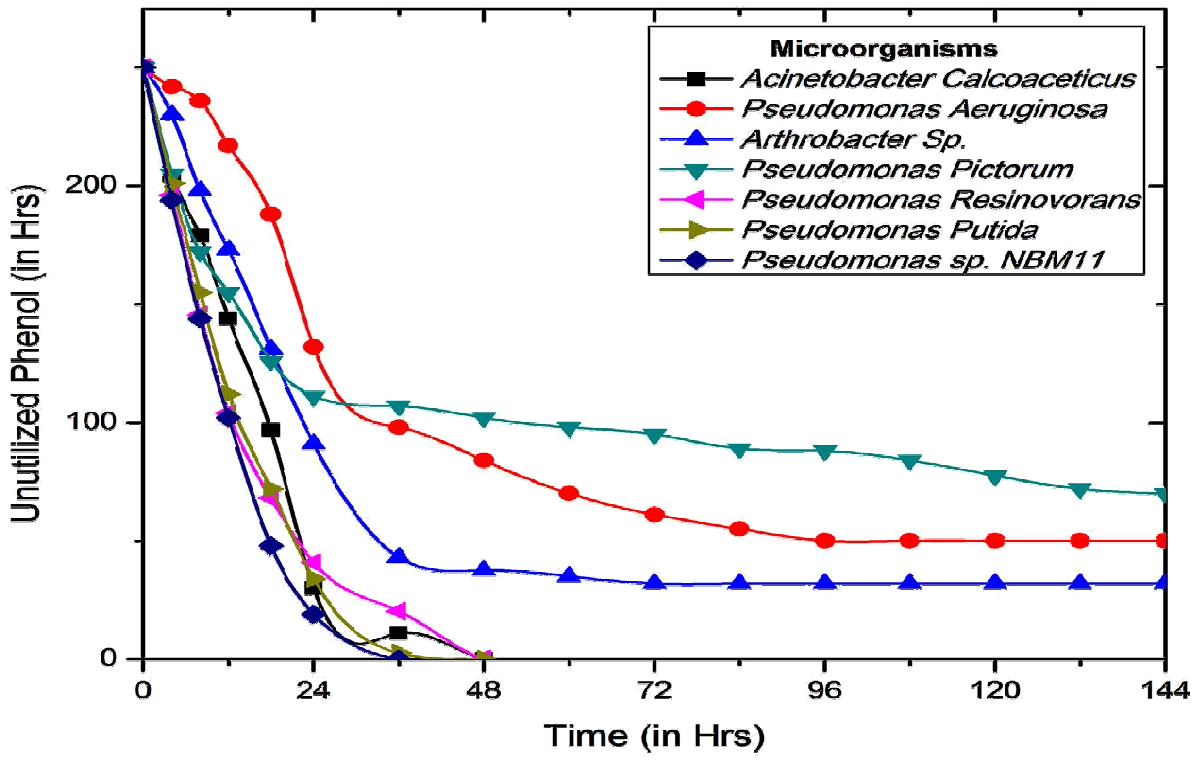

FIGURE 3.23: COMPARISON OF THE DEGRADATION POTENTIAL OF VARIOUS MICROBES AT 250 PPM OF PHENOL(30°C, pH 7)

3.7.2 AT 500 PPM OF INITIAL CONCENTRATION OF PHENOL

Figure 3.24 shows the degradation profile of the microbes at 500 ppm of initial concentration of phenol. *Pseudomonas sp., NBM11* degrades phenol completely in 84 hours while *Pseudomonas putida* degrades in 96 hours, *Acinetobacter calcoaceticus* and *Pseudomonas resinovorans* degrades the same concentration of phenol in just 72 hours. Most of the microbes except *Pseudomonas pictorum* are able to degrade the substrate below the permissible limit while *Pseudomonas resinovorans* is able to degrade the substrate completely within 120 hours.

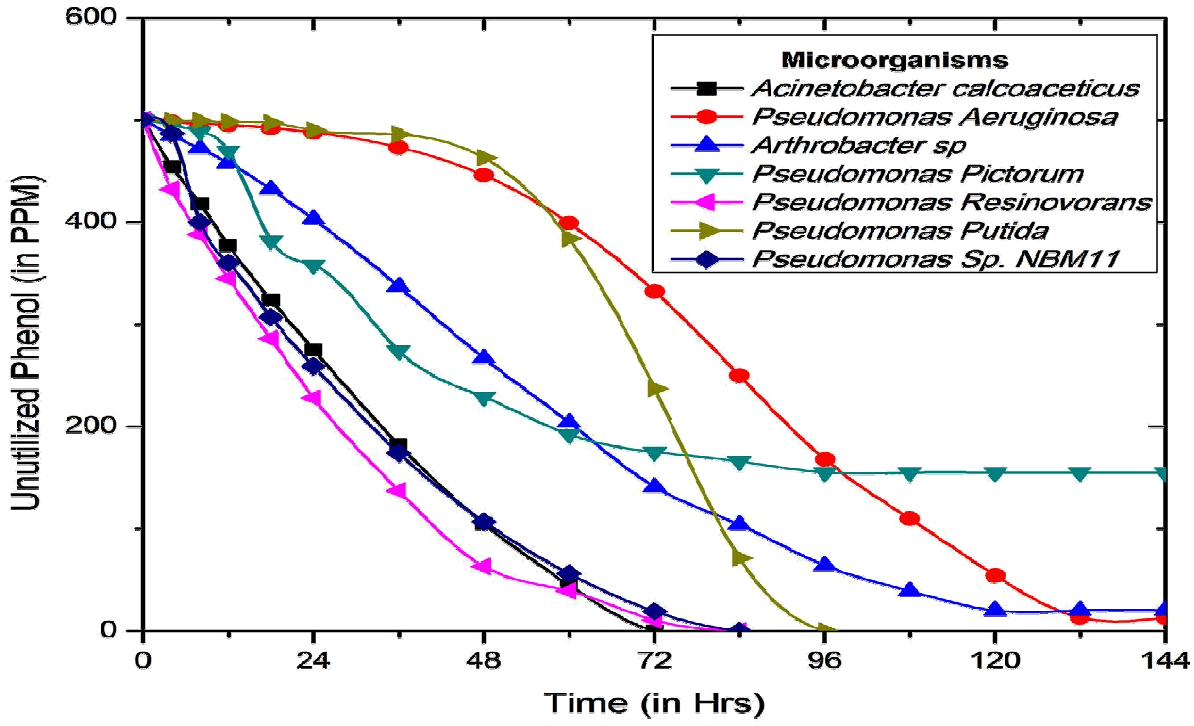

FIGURE 3.24: COMPARISON OF THE DEGRADATION POTENTIAL OF VARIOUS MICROBES AT 500 PPM OF PHENOL (30°C, pH 7)

3.7.3 AT 750 PPM OF INITIAL CONCENTRATION OF PHENOL

Figure 3.25 represents the degradation curve of the microbes at the initial concentration of phenol of 750 ppm. The microbes started showing a characteristics lag phase for the increased toxicity of the substrate concentration. But still the isolate and *Acinetobacter calcoaceticus* are able to degrade the substrate completely in 120 and 96 hours respectively. While *Pseudomonas putida* and *Pseudomonas resinovorans* degrades the complete substrate in 144 hours and 120 hours respectively the rest microbes are unable to

completely utilize the substrate. At such high concentration the degradation activity of the microbe *Pseudomonas resinovorans* is better than the rest of the microbes.

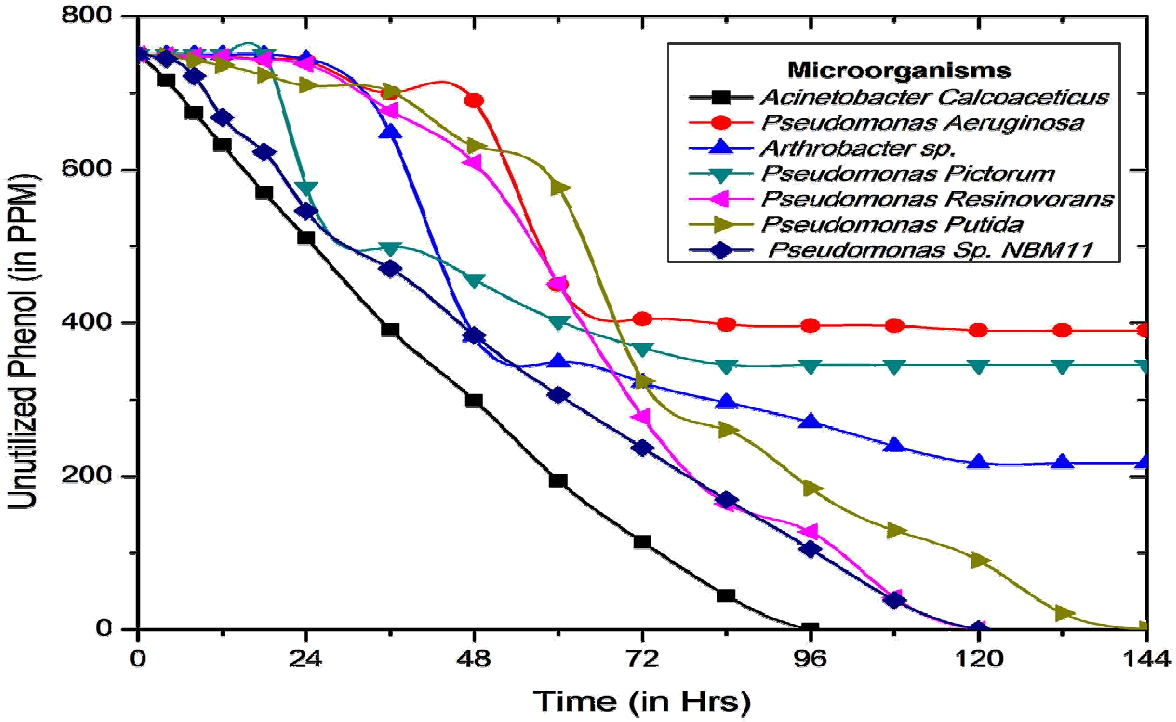

FIGURE 3.25: COMPARISON OF THE DEGRADATION POTENTIAL OF VARIOUS MICROBES AT 750 PPM OF PHENOL (30°C, pH 7)

3.7.4 AT 1000 PPM OF INITIAL CONCENTRATION OF PHENOL

While most of the microbes were unable to grow at such high concentration of phenol certain microbes like *Arthrobacter Sps*., and *Pseudomonas pictorum* are able to survive the concentration but unable to degrade the substrate since a increased substrate concentration decrease their activity. Only two of the reference microbes (*Acinetobacter calcoaceticus* and *Pseudomonas putida*) taken in the study were able to degrade such high concentration of phenol. But the isolate obtained in the present study is also able to degrade 1000 ppm of phenol in 168 hours and the degradation profile is much better than the *Pseudomonas putida* as evidenced from the figure 3.26. Even if all the three microbes takes the same 168 hours for the complete mineralization of the substrate but on the basis of the degradation pattern of the microbes it can be concluded that *Pseudomonas sp.* NBM11 shows a better degradation profile than *Pseudomonas putida*.

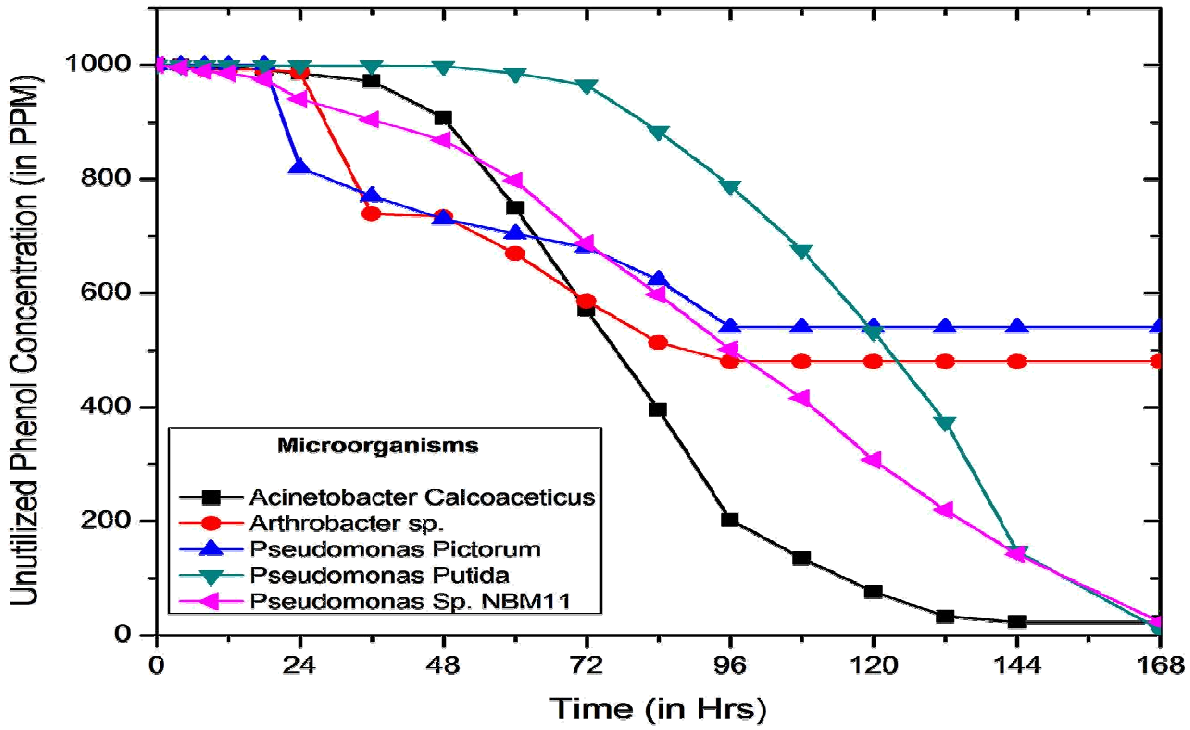

FIGURE 3.26: COMPARISON OF THE DEGRADATION POTENTIAL OF VARIOUS MICROBES AT 1000 PPM PHENOL (30°C, pH 7)

3.8 DETERMINATION OF THE BIOKINETIC PARAMETERS

The biokinetic parameters of the microbe *Pseudomonas sp.* NBM11 has been estimated by fitting the growth data to Monod Kinetic Model. The maximum specific growth (μ_{max}) and half saturation constant (K_s) has been determined for each initial concentration of phenol. It has been observed that with increase in initial concentration of phenol the μ_{max} decreases owing to toxic nature of the substrate. The plots were obtained using Origin plotting software and the kinetic parameters μ_m , K_s were obtained using the equations given in section 2.8. For each microorganism, different μ_m, K_s value is obtained and were listed in the table 3.4.

In this study phenol is used as the growth limiting factor since it is the sole source of carbon and energy. But even if it is a sole source for the microbe its increasing concentration is toxic for the microorganism. Hence instead of increasing, the specific growth rate of the microbe decreases with each unit of increase in concentration. From the table 3.4 it can be noted that with increasing concentration of the substrate the maximum specific growth rate

decreases and the half saturation constant increases. After a certain point the half saturation constant for the microorganism increases drastically.

TABLE 3.4 : BIOKINETICS PARAMENTERS OF THE DIFFERENT MICROORGANISM AT VARIOUS INITIAL CONCENTRATION OF PHENOL.

Microorganism	***Concentration\ (in PPM)***	***Maximum Specific Growth(μ_{max}) (in hr^{-1})***	***Half Saturation Constant (K_s) (mg $L^{-1}hr^{-1}$)***
Acinetobacter calcoaceticus	250	0.06	4.22
	500	0.017	46
	750	0.006	63.17
	1000	0.005	71.68
Pseudomonas putida	250	0.035	11.71
	500	0.014	17.6
	750	0.012	116
	1000	0.011	331.98
Pseudomonas aeruginosa	250	0.0624	61.442
	500	0.054	350.488
	750	0.013	419.395
	1000	----------	----------
Arthrobacter sps	250	1.282	32.614
	500	0.78125	171.093
	750	0.438	235.644
	1000	0.046	409.814
Pseudomonas pictorum	250	0.298	71.044
	500	0.1173	111.476
	750	0.0234	142.99
	1000	0.0138	755.502
Pseudomonas resinovorans	250	0.061	13.92
	500	0.012	14.46
	750	0.005	93
	1000	----------	----------
*Pseudomonas sp.*NBM11	250	0.048	9.87
	500	0.016	20.17
	750	0.012	76.37
	1000	0.011	162.35

As suggested by Agarry et al. (2010), phenol is an inhibitory substrate with increase in its concentration the specific growth rate decreases and as a result we find the above pattern of the graph. Hinteregger et al. (1992) have reported similar observations.

TABLE 3.5 : BIOKINETICS PARAMENTERS OF THE DIFFERENT MICROORGANISM AS REPORTED BY LITERATURE

MICROORGANISM	REFERENCE	CONCENTRATION OF PHENOL(mg/L)	$\mu_{MAX}(hr^{-1})$	K_S(mg/L)
Acinetobacter calcoaceticus	Kumaran & Pachuri (1997)	60-500	0.465	30.96
Pseudomonas putida	Kumar et al. (2005)	0-1000	0.216	20.59
Pseudomonas aeruginosa	Agarry et al. (2010)	500	NA	331.2
Arthrobacter sp.	Kar et al.(1997)	500	0.8	NA

The maximum specific growth rates of the subjected microbes obtained in the present study does not falls within the range of the maximum specific growth rates reported by the literature available. The probable reason behind this difference in the obtained results may be attributed to the difference in the experimental conditions method of evaluation. Moreover sufficient data is not available regarding the biokinetic parameters of *Pseudomonas pictorum* and *Pseudomonas resinovorans*. It can be observed that the difference in the maximum specific growth values obtained in the present study and the available literature is too high and the reason behind it is the use of additional carbon source along with phenol. In the current study, phenol is used as the sole source of carbon and energy and as mentioned earlier it is toxic in nature and confers certain toxicity to the microorganism. While in the available literature, it was observed that most of the experiments were carried out in the media containing additional carbon and nitrogen sources like glucose and peptone. Hence growth of microorganism in these medium will be more profuse as compared to the medium deficient of these additional sources resulting in a lower value of maximum specific growth exhibited by the microorganisms in the same concentration of phenol.

Kotturi et al. (1991) have noted that the half saturation coefficient is influential on the growth kinetics in low concentration region. At the same time, the small changes in the

biomass and substrate concentrations in batch reactors cannot be measured accurately. Therefore, this may be one of the possible reasons for the discrepancy in values of half saturation coefficient, *K*s. It may be mentioned that Hill and Robinson (1975) determined the half saturation coefficient using continuous flow mixed reactor. The dilution rate and effluent substrate concentration data are required to calculate the value of *K*s. The dilution rate and effluent substrate concentrations can be relatively measured accurately.

3.9 PHENOL DEGRADATION BEHAVIOR OF IMMOBILIZED MICROORGANISM

Biodegradation of phenol has been studied in detail using both pure and mixed cultures of suspended bacteria. However, at higher concentrations of phenol microbial growth gets inhibited. Hence several strategies have been proposed by various researchers to overcome substrate inhibition. Immobilized cell technology has been widely applied in a variety of research and industrial applications. The degradation of aromatic compounds by immobilized cells has been reported and much work has been carried out on immobilized cell reactors using phenol as the model toxic compound. Immobilized cells offer the possibility of degrading higher concentrations of toxic pollutants than can be achieved with free cells. It has been shown by several workers that immobilized microorganisms are better protected against phenolic compounds than are free cells. Various support matrix, such as activated charcoal, calcium alginate beads, PAA, chitosan etc were been used for the immobilization of microbial cells. Continuous degradation of phenol with immobilized cells have been attempted by Ehrhardt and Rehm (1989) and Morsen and Rehm (1990).

Most of the researcher have worked on the immobilization of the microbe by adsorption on activated charcoal and a very few have opted for the other alternatives like calcium alginate or PAA. A great advantage of activated carbon is the ability to work as a buffer by adsorbing high concentrations of phenol and, thus, protect the adsorbed microorganisms against damage by phenol (Morsen and Rehm, 1990). Biomass built up on activated carbon during continuous degradation of phenol was found to be six times higher than on sintered glass beads (Morsen and Rehm, 1990).

While numerous immobilization techniques have been described, entrapment within synthetic or natural polymers remain the most popular due to its ease and simplicity, low

cost and gentle formulation conditions ensuring high retention of cell viability. The most popular approach for whole cell immobilization is gel entrapment, e.g. in Ca-alginate or carrageenan gels (Freeman, 1994).

The main advantage of gel immobilization is the biocompatibility; although large-scale formulation is difficult, the beads are often permeable to cells, mass transfer limitations are often encountered, and the reactor volume occupied by the beads is generally significant. The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes. It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. The ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very high dilution rates, are few of the many advantages of immobilized cell systems. The metabolically active cell immobilization is particularly preferred where co-factors are necessary for the catalytic reactions. Since co-factor regeneration machinery is an integral function of the cell, its external supply is uneconomical. Hence, an attempt has been made to immobilize cells of microbes and test their phenol degradation efficiency in the present study.

Pai et al. (1995) studied the immobilization of Rhodococcus Sps. on both activated carbon and calcium alginate to evaluate the benefit of immobilization by entrapment. They have reported that when the initial concentration phenol is higher than 1200 PPM the adsorption of microbe on activated charcoal but they reported that when initial concentration of phenol is below 1200 PPM entrapment of microorganism in alginate bead yielded better result. It is so because the resistance of alginate bead towards phenol transfer is low and hence at the concentration when substrate is inhibitory to the microbe, entrapment yields poor result. But it does not inhibit the degradation completely. Thus in the present study we opted for calcium alginate for the immobilization of the microbes for the enhancement in their biodegradation potential

Figure 3.27 represents the degradation profile of the *Acinetobacter calcoaceticus* immobilized in calcium alginate bead. The microbe as reported by the literature (Abd-El-Haleem et al., 2003) is able to degrade 500 PPM of phenol within 20 hrs while it takes less

than 50 hrs to degrade 1000 PPM of phenol. The immobilized cells have higher degradation rate and is tolerable to wider initial phenol concentration in the medium (Ying et al., 2007). Similar results were obtained by Beshay et al. (2002) where the immobilized Acinetobacter Sps., degraded 500 PPM of initial concentration of phenol in 40 hrs while the free cells took 120 hours to degrade the same concentration of phenol.

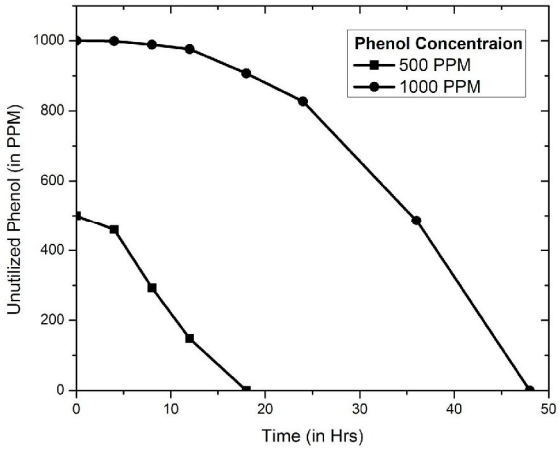

FIGURE 3.27: DEGRADATION CURVE OF IMMOBILIZED ACINETOBACTER CALCOACETICUS (30^{o}C, pH 7)

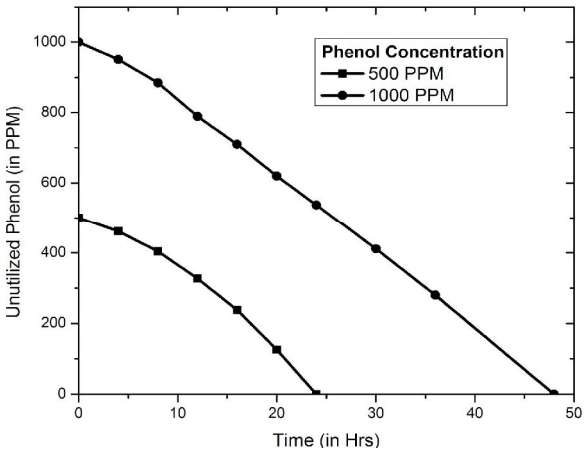

FIGURE 3.28: DEGRADATION CURVE OF IMMOBILIZED PSEUDOMONAS PUTIDA (30^{o}C, pH 7)

Figure 3.28 represents the degradation curves of the immobilized *Pseudomonas putida* in various initial concentration of phenol. The microbe is able to degrade 500 PPM of phenol within 24 hours while it degrades1000 PPM of phenol in just 50 hours. Gonzalez et al. (2001) have reported that the batch phenol biodegradation experiment on the calcium alginate immobilized *Pseudomonas putida* yield similar results where the immobilized microorganism degraded 500 PPM of phenol in 25 hours. Bandyopadhyay et al. (2001) have studied the biodegradation of phenol by *Pseudomonas putida* immobilized in calcium alginate and reported that on increasing the concentration of the phenol above 750 PPM the reaction behavior deviates from michelean kinetics which may be due to the effect of intraparticle diffusion.

Figure 3.29 shows the degradation curve of the immobilized *Pseudomonas aeruginosa* at different initial concentration of phenol. The microbe is able to degrade 500 PPM of phenol in just 30 hours while it completely degrades1000 ppm of phenol within 70

hours. Puerto telo et al. (2009) have compared the biodegradation behavior of *Pseudomonas aeruginosa* immobilized on two different types of activated carbons and reported that the immobilized microbe is able to degrade up to 972 PPM of phenol from the initial concentration of 1000 PPM in a 3 days' time. They also reported that agitation enhances the biodegradation activity of the microbe.

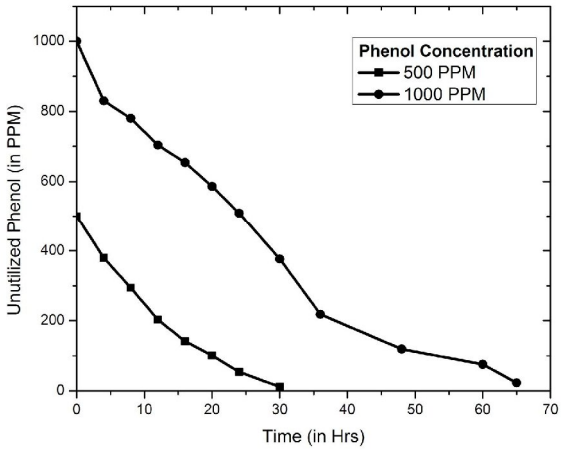

FIGURE 3.29: DEGRADATION CURVE OF IMMOBILIZED PSEUDOMONAS AERUGINOSA (30°C, pH 7)

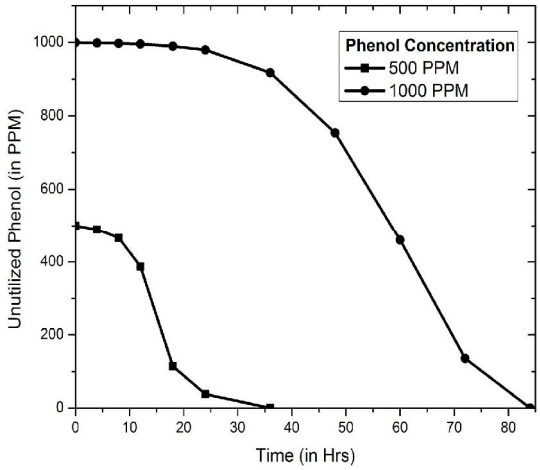

FIGURE 3.30: DEGRADATION CURVE OF IMMOBILIZED PSEUDOMONAS PICTORUM (30°C, pH 7)

Figure 3.30 represents the degradation curve of immobilized *Pseudomonas pictorum* at different initial concentration of phenol. As seen from the Figure, the microbe is able to degrade 500 PPM of phenol within 40 hours while it has the ability to degrade 1000 PPM of phenol within 84 hours. Sheeja and Murugeshan (2002) have reported that on immobilizing *Pseudomonas pictorum*, the microbe is able to degrade up to a concentration of 2000 PPM. While the free cells of the bacterial strain is unable to tolerate more that 1000 PPM of the substrate. They also tried a different type of immobilization technique in which they immobilized the microbial strain along with activated charcoal in calcium alginate beads. Annadurai et al. (2000) reported that *Pseudomonas pictorum* immobilized on activated charcoal is an excellent possibility for the reduction of the cost of treatment of phenol. They reported that the immobilized system is able to degrade up to 99% of initial concentration of phenol till 600 PPM.

On immobilization, it was observed that Arthobacter sps., is able to degrade up to 1000 PPM of phenol within 60 hours while it degrades the substrate considerably below the detection limit within 20 hrs when the initial concentration of phenol is 500 PPM as depicted by Figure 3.31. Karigar et al. (2006) have immobilized the strain Arthrobacter citreus in agar as well as calcium alginate and found that the immobilized microbe is able to degrade up to 22mM of phenol in a time period of 8 days. They also have shown in their study that the microbe immobilized in calcium alginate beads shows better biodegradation profile as compared to the microbe on immobilization in agar.

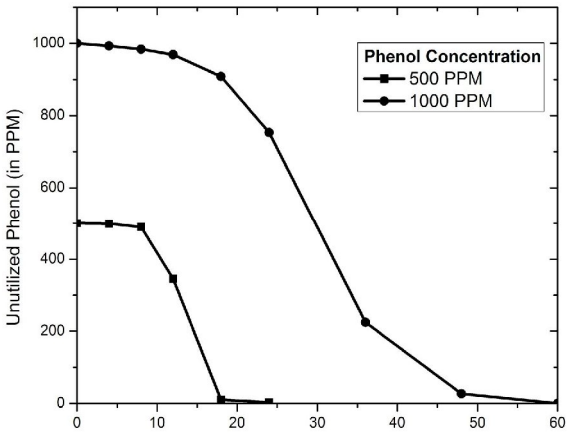

FIGURE 3.31: DEGRADATION CURVE OF IMMOBILIZED ARTHROBACTER SPS (30°C, pH 7)

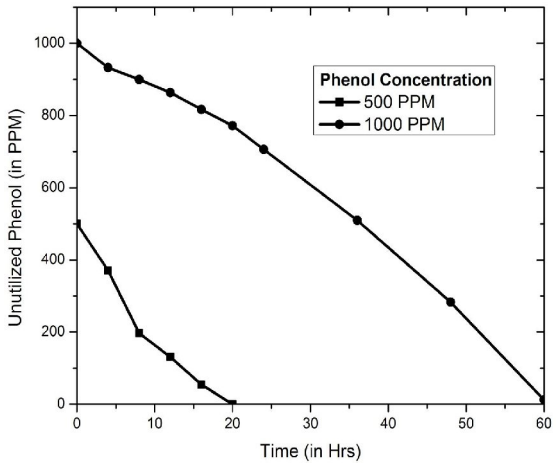

FIGURE 3.32: DEGRADATION CURVE OF IMMOBILIZED PSEUDOMONAS RESINOVORANS (30°C, pH 7)

Till date not much work has been done on the biodegradation potential of the microbe *Pseudomonas resinovorans*. Hence the biodegradation of the cells immobilized in any immobilizing agent is yet to be studied. For the first time, in this study, degradation potential of the cells immobilized in ca-alginate beads at various concentration of phenol has been reported. The result from the study is reported in figure 3.32. The microbe is able to degrade 500 PPM of phenol in just 20 hours. Complete degradation of the substrate when the initial concentration of the phenol is 1000 ppm is observed within 60hours. Thus the present study suggests that the microbe *Pseudomonas resinovorans* can be used for the efficient biodegradation of phenol in waste water treatment when immobilized in any immobilizing agent.

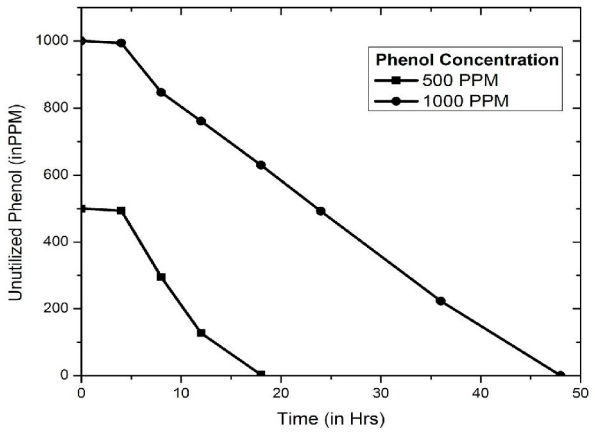

FIGURE 3.33: DEGRADATION CURVE OF IMMOBILIZED ISOLATE PSEUDOMONAS SP. NBM11 (30°C, pH 7).

Figure 3.33 suggest the phenol biodegradation potential of the isolate *Pseudomonas sp.* NBM11 at different concentration of phenol. In the present study the isolate obtained from the contaminated site has been immobilized on the ca-alginate bead and the biodegradation potential of the microbe is observed. Figure 3.38 reveals that the microbe is able to degrade 1000 PPM of phenol within 50 hours while it is able to degrade 500 PPM of initial phenol within 20 hours. Thus it can be said that the isolated microbe is a potential biodegrading agent when immobilized.

3.10 COMPARISON OF PHENOL DEGRADING CAPACITY OF THE BOTH FREE CELL AS WELL AS IMMOBILIZED MICROROGANISM

In the present study, the biodegradation potential of both isolated and reference microorganism in immobilized and non- immobilized form has been compared. This study has been carried out to record the change in the degradation profile of the microbes on immobilizing them in calcium alginate bead. Bettmann and Rehm (1984) immobilized *Pseudomonas sp.* into alginate and polyacrylamide hydrazide (PAAH). The immobilized *Pseudomonas sp* was able to degrade phenol at an initial concentration of up to 2000 PPM in less than two days, although the free cells did not grow at this concentration. Ehrhardt and Rehm (1985) studied phenol degradation by *Candida* sp and *Pseudomonas* sp. immobilized on activated carbon. They found that the free cells of *Candida* sps did not tolerate more than 1500 PPM phenols. But the immobilized cells tolerated phenol concentration up to 15000 PPM and degraded about 90% of phenol within 200 hours. Thus a large change in

biodegradation potential of the microbes is observed when immobilized in certain immobilizing agent. Hence to evaluate the behavioral changes in the microbes' biodegradation capability on entrapment, the degradation profile of both freely suspended and immobilized cells were compared.

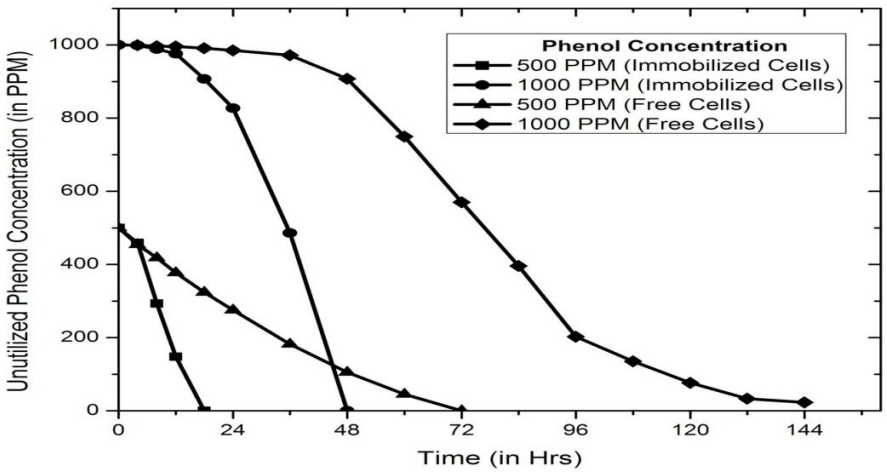

FIGURE 3.34: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED CELLS OF ACINETOBACTER CALCOACETICUS (30^0C, pH 7).

Figure 3.34 represents the comparative study of the biodegradation potential of the *Acinetobacter calcoaceticus* in both immobilized and free cell form. From the above Figure it can be concluded that the microbe is able to degrade up to 1000 ppm of phenol as an initial concentration but the time taken for degradation changes thus indicating that the rate of degradation is increasing. While the suspended cells are able to degrade the substrate of concentration of 500 PPM in 72 hours the same cells when immobilized degrades the same concentration of the substrate in just 20 hours. While in freely suspended form *Acinetobacter calcoaceticus* is able to degrade 1000 ppm of phenol in 144 hours the same cells on immobilization degrades same concentration of substrate in just 48 hours.

Figure 3.35 depicts the comparison of the phenol biodegradation potential of the *Pseudomonas putida* in both freely suspended form and cells immobilized in ca-alginate beads. It is affirmed that the microbe degrades 500 ppm of initial phenol in just 24 hours when immobilized while it takes 84 hours to degrade same concentration of phenol in its freely suspended form.

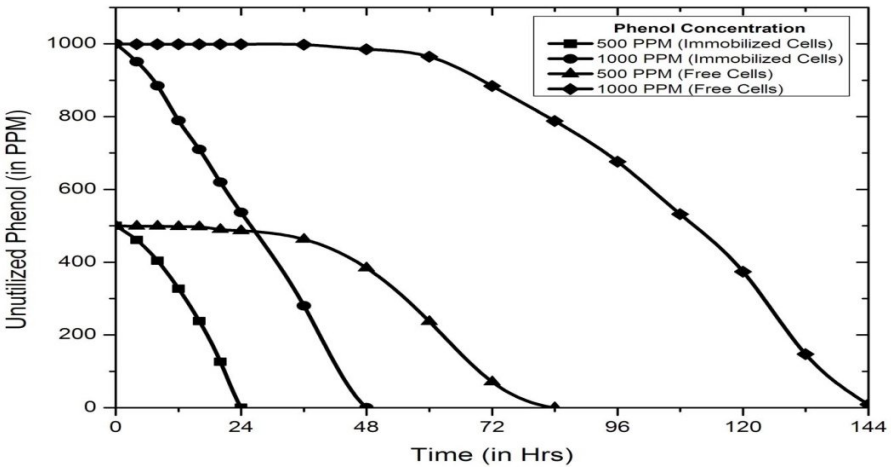

FIGURE 3.35: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED CELLS OF PSEUDOMONAS PUTIDA (30^{o}C, pH 7)

From the figure we can also note that the microbe is able to degrade 1000 ppm of phenol in just 48 hours while the free cells degrade the same concentration of phenol in 144 hours. A prolonged lag phase has been observed in the degradation curve of the microbe in its freely suspended form but on being immobilized no such lag phase is observed. This may be due to the fact that degradation is indirectly proportional to the growth of the microbe and tolerance to the stressed environment. Since phenol is a growth limiting substrate, the increase in concentration of phenol increases the lag phase due to its toxicity. But on entrapment the microbe is not exposed directly to the stress environment and as a result of which it is able to utilize the substrate faster and in a uniform pattern.

Pseudomonas aeruginosa was not able to grow at a concentration higher than 750 ppm of phenol in the free cell form as discussed earlier. But on immobilization, it is found that the microbe is able to tolerate up to 1000 ppm of phenol and is able to degrade such high concentration of phenol in just 72 hours. Thus figure 3.36 represents the behavioral change in the phenol degradation curve of *Pseudomonas aeruginosa* on immobilization. Another factor that can attribute to the degradation of such high concentration of phenol is that at when the phenol is used as the sole source of carbon and at such high concentration, there is no growth of the biomass and hence no degradation. But when the cells are immobilized in the protectant the substrate is unable to confer its toxicity on the microbe and it utilizes the phenol for its metabolism.

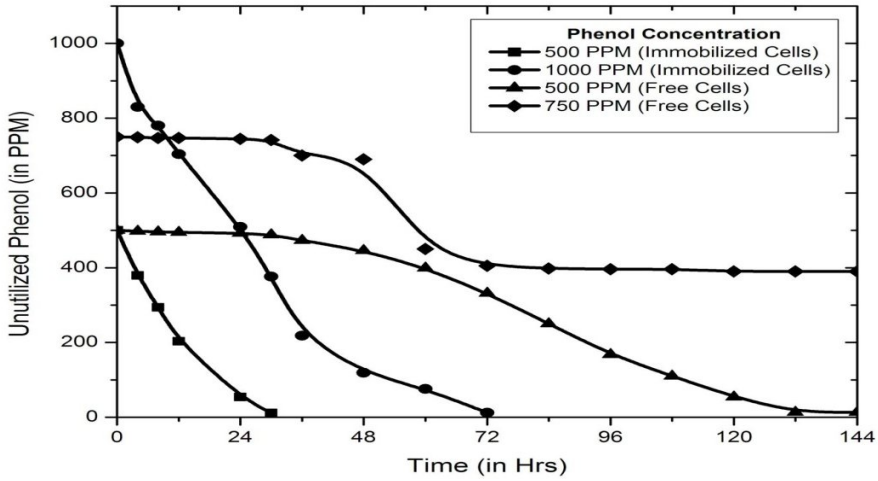

FIGURE 3.36: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED CELLS OF PSEUDOMONAS AERUGINOSA (30^{o}C, pH 7)

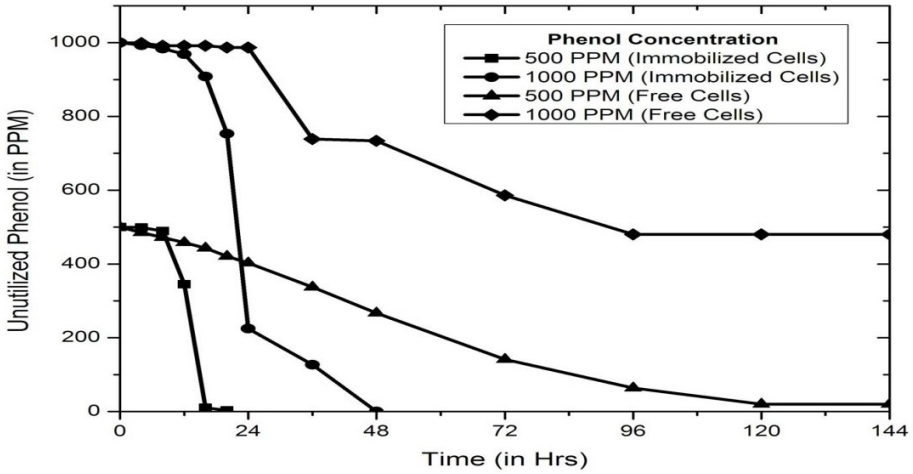

FIGURE 3.37: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED CELLS OF ARTHROBACTER SPS. (30^{o}C, pH 7)

Figure 3.37 it is observed that *Arthrobacter Sps.* is able to tolerate up to 1000 PPM of phenol but is unable degrade it completely below the detection limit. But on entrapping it in alginate beads the microbes takes less time to utilize the substrate completely. Figure 3.37 indicates that the microbe is able to degrade only 52 % of phenol when the initial concentration was 1000 PPM as free cell but on immobilization the microbe degrades the substrate of same concentration completely within 48 hours. Similarly when the initial concentration of phenol is 500 PPM the microbe is able to degrade 96% of the initial concentration of phenol in 144 hrs in its freely suspended form while it degrades the same

concentration of phenol completely in just 24 hours when immobilized in ca-alginate beads. Thus the efficiency of the phenol degradation increases with immobilization.

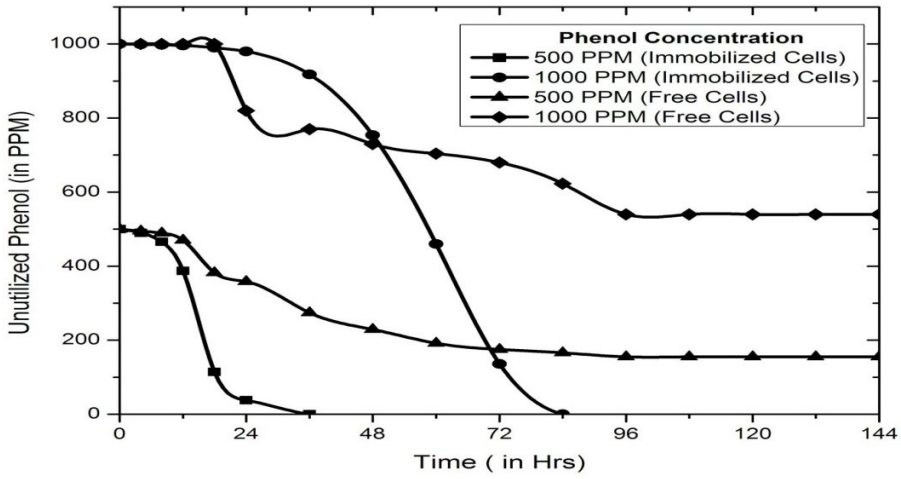

FIGURE 3.38: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED CELLS OF PSEUDOMONAS PICTORUM (30°C, pH 7).

Pseudomonas pictorum shows a marked change in the degradation pattern of phenol which can be observed from the figure 3.38. When the initial concentration of the phenol in the media is 500 ppm the freely suspended cells were able to degrade 69% of initial concentration in 144 hours while the immobilized cells are able to degrade complete substrate in just 36 hours. Similarly when the initial concentration of phenol is 1000 ppm, the freely suspended form of the microbe is able to degrade only 46% of the initial concentration while the immobilized cells were able to degrade the substrate completely within 84 hours. Thus it can be concluded that immobilization increases the degradation potential of the microbe. The maximum phenol biodegradation potential of the *Pseudomonas resinovorans* is 750 ppm phenol as free cells. When the cells are immobilized in ca-alginate beads the tolerance of the microbe increases.

Figure 3.39 represents the comparison in the biodegradation behavior of the strain *Pseudomonas resinovorans* in both free cell as well immobilized form. The microbe degrades 500 ppm of phenol in just 20 hours when immobilized in calcium alginate beads while in its free cell form the microbe takes 72 hours to degrade the same concentration of completely. Moreover immobilization not only increases the tolerance but also the

degradation efficiency of the microbe and makes it a suitable candidate for the biodegradation.

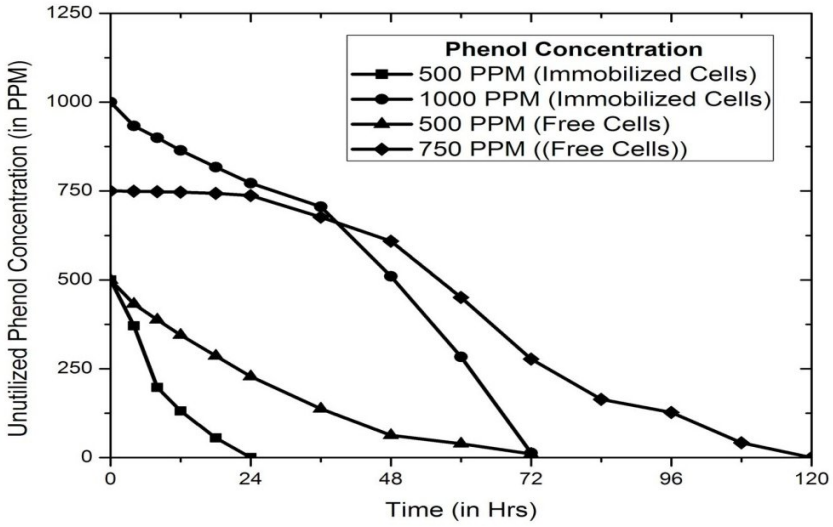

FIGURE 3.39: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED PSEUDOMONAS RESINOVORANS (30^{o}C, pH 7)

The microbe is able to degrade 1000 ppm of phenol in just 60 hours while in the freely suspended-form the microbe takes 120 hours for the complete degradation of 750 ppm of phenol and a concentration of 1000 PPM was lethal to the microbe.

The microbe isolated in the present study *Pseudomonas sp.* NBM11 has been subjected to immobilization and the comparative analysis of the phenol degrading potential of the microbe in its freely suspended cell form and immobilized form is depicted in the Figure 3.40. As discussed earlier the microbe is able to degrade 500 and 1000 ppm of initial concentration of phenol in 84 and 168 hours respectively in its freely suspended cell form. But on immobilization in ca-alginate bead the microbe is able to degrade the same initial concentration of phenol in 20 and 48 hours respectively. Hence it can be seen that there is a marked change in the degradation efficiency of the isolate. The microbe *Pseudomonas sp.*NBM11 is able to tolerate and degrade up to 1000 ppm of phenol but its efficiency can be increased for its better utilization in different wastewater treatment plant by immobilizing in any immobilizing agent.

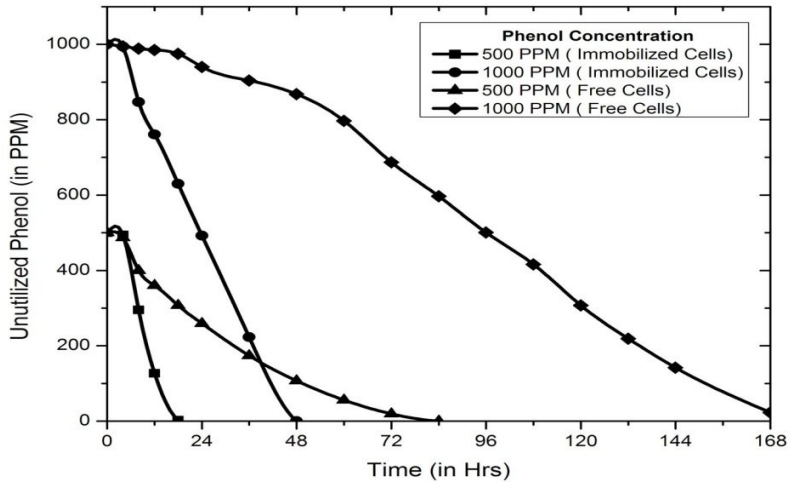

FIGURE 3.40: DEGRADATION CURVE OF FREE CELL VS IMMOBILIZED FORM OF ISOLATE PSEUDOMONAS SP. NBM11 (30^{o}C, pH 7)

3.11 IDENTIFICATION OF THE CATABOLIC GENES RESPONSIBLE FOR PHENOL DEGRADATION

There are reports on many microorganisms that are capable of degrading through the action of various enzymes. The critical step in the degradation of the aromatic compounds is hydroxylation of the xenobiote. Based on the initial product of the ring cleavage by the enzyme, the pathway followed for the degradation of the substrate is grouped as catechol pathway, gentisate pathway and protocatecuate pathway. The ring fission products are then transformed leading to the metabolism of the microbe. Most of the microbes degrade phenol by catechol pathway. Aerobically, phenol also is first converted to catechol, and subsequently, the catechol is degraded via ortho or meta fission to intermediates of central metabolism.

Out of all the subjected microbial strains, *Pseudomonas putida* and *Acinetobacter calcoaceticus* were been studied exhaustively and they both were reported to have phenol hydroxylase gene. Schirmer et al. (1997) reported the presence of phenol hydroxylase and catechol-1.2-dioxygenase encoded by *mop* operon in *Acinetobacter calcoaceticus*. Expression of the *mop* operon depends on σ54 RNA polymerase and is induced by phenol (Ehrt et al., 1995). Similarly, phenol hydroxylases together with the genes encoding the *meta* cleavage pathway of catechols are organized in operons located on the TOL plasmid (Powlosky et al., 1990) or the chromosome of *Pseudomonas* spp. (Shingler et al., 1993).

Takeo et al. (1995) cloned and sequenced the multicomponent phenol hydroxylase gene *pheA* in *Pseudomonas putida BH*.

Several known sequences of the components of phenol hydroxylase from the available literature were used to design primers to amplify the sequence of the DNA that encodes the enzyme. For enhancement of the expression of the phenol hydroxylase genes, microbes were grown on phenol. The objective of the present study is to trace the catabolic phenol hydroxylase or monoxygenases genes that are responsible for the degradation of phenol by the microbes. Four primers which were usually reported for the amplification of phenol hydroxylase and phenol monoxygenases genes in the microbes were named as PH, LmpH, DmpN and PMO. Each microorganism i.e. *Pseudomonas pictorum*, *Pseudomonas aeruginosa*, *Pseudomonas resinovorans*, *Arthrobacter Sps*. and the isolate *Pseudomonas Sp.* NBM11 were subjected to PCR amplification using the above mentioned four primers. Since much work has been done on *Pseudomonas putida* and *Acinetobacter calcoaceticus*, these strains were refrained from this study.

Figure 3.41, suggests that the DNA amplified with the PH primer (Cafaro et al., 2004) resulted in positive bands in two microbial strain. The expected size of the PCR product was 267 bp. Lane 2 is the positive control of the *Pseudomonas stutzeri*. Lane 4 is the amplification of the gene from *Pseudomonas pictorum* and lane 5 is the amplicon obtained from *Pseudomonas aeruginosa*. Both the microbes were reported to be having the ability to degrade phenol but till date the genes responsible for the same have not been explored by the scientific community. Amplification of a 287 bp band with the primer PH-m indicates the presence of the multicomponent phenol hydroxylase enzyme which is responsible for the degradation of phenol. Cafaro et al. (2004) reported the presence of the gene phenol hydroxylase (PH) in *Pseudomonas stutzeri* OX1. They have reported the presence of two components in the phenol hydroxylase enzyme, PHM and PHP. Primers *PHMup* and *PHMdown* were used for the amplification of a 267-bp fragment containing the *ph-m* gene, coding for PH M. They reported that PH P component of the phenol hydroxylase enzyme transfers electron from NADH to a subcomplex endowed with hydroxylase activity while regulatory functions were assigned for the PH M component.

Hence from the figure 3.41 and the investigations suggested by Cafaro et al. (2004), it is concluded that the microbial strains *Pseudomonas aeruginosa* and *Pseudomonas*

pictorum used in this study contains the phenol hydroxylase gene PH which has been reported in *Pseudomonas Stutzeri* OX1. The phenol hydroxylase PH is a multicomponent phenol hydroxylase enzyme which belongs to group 1 bacterial multicomponent monoxygenases. They also reported that the component PHM is a very important component of the phenol hydroxylase enzyme and even if it cannot activate the phenol biodegradation by itself, on being complexed with the PHP subunit of the phenol hydroxylase enzyme increases the activity of the enzyme.

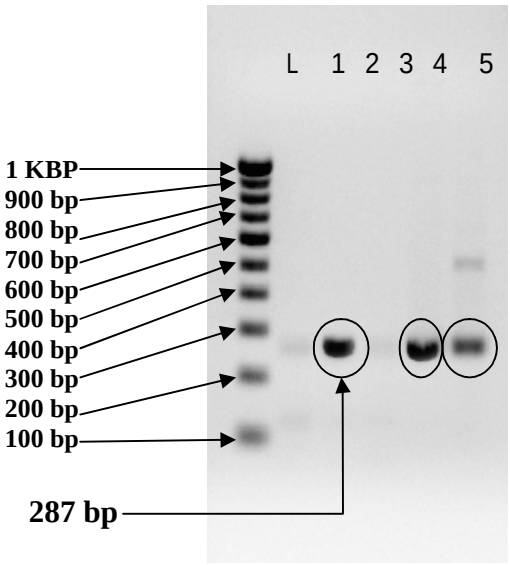

FIGURE 3.41: AMPLIFICATION OF PHENOL HYDROXYLASE GENE FROM PSEUDOMONAS PICTORUM.

LmpH is the largest subunit of the multi-component phenol hydroxylase enzyme (Futamata et al., 2001). It has three phylogenetic groups named class I, II, and III based on their kinetic constants. The primer has been designed from the sequence reported by Shingler et al., (1992) of primers which were used in different combination to obtain the required gene of class. This universal primer suggests that it has been designed from the consensus sequence of a number of phenol hydroxylase enzyme obtained from different microbes that were been known for their phenol degrading potential. Futamata et al., (2001) reported that primer which amplifies the LmpH class I enzyme, designed to give a amplicon size of 600 bp while the LmpH class II enzyme amplifies a 400 bp of amplicon.

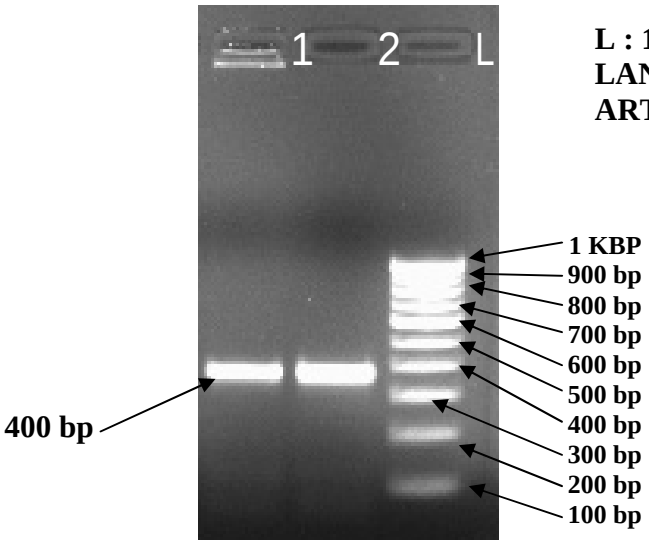

FIGURE 3.42 AMPLIFICATION OF PHENOL HYDROXYLASE GENE LMPH (400 BP) FROM ARTHROBACTER SPS

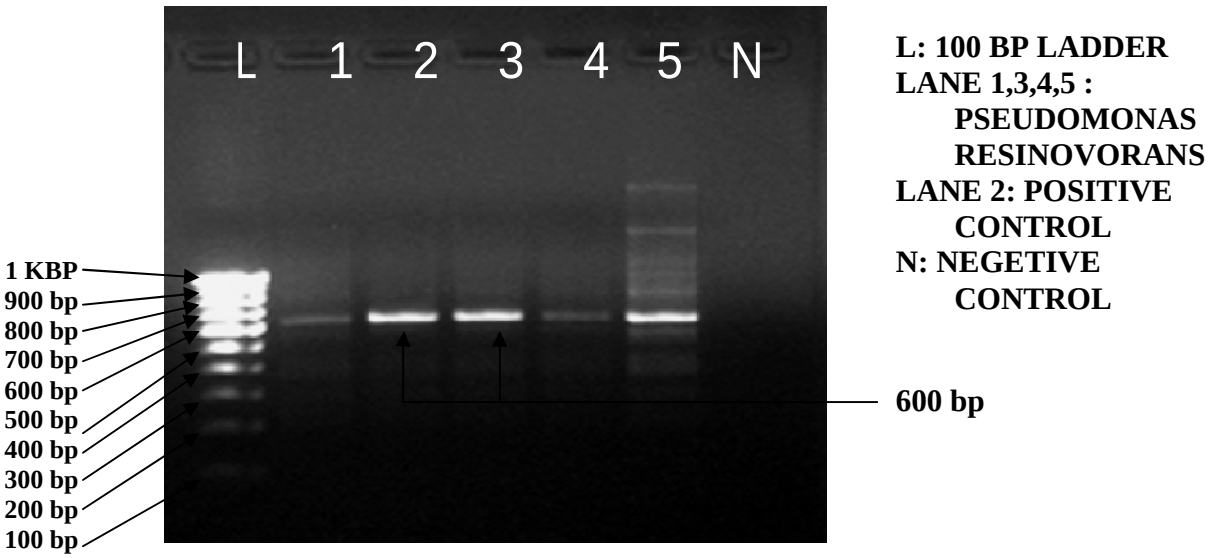

FIGURE 3.43: AMPLIFICATION OF PHENOL HYDROXYLASE GENE PHE U (600 BP) FROM PSEUDOMONAS RESINOVORANS.

Figure 3.42 indicates that microbial strain *Arthrobacter Sps*. used in this study shown a distinct band around 400 bp from the primers designed to amplify the LmpH class III genes. The obtained results along with the available literature suggests that that *Arthrobacter sps*., contains LmpH class III gene which is responsible for the utilization of phenol as mentioned by Futamata et al. (2001).

Figure 3.43 is the gel image of the PCR product of the DNA obtained from *Pseudomonas resinovorans* amplified with Phe U primer. Phe U primer is designed to amplify the group I LmpH, phenol hydroxylases. As suggested by Futamata et al. (2001) and Movahedyan et al. (2009), the DNA amplifies a 600 bp amplicon from the designed primer which indicates the presence of the phenol hydroxylase gene LmpH. Thus the study revealed the presence of the phenol hydroxylase gene which is responsible for the efficient degradation of the phenol by the bacterial strain.

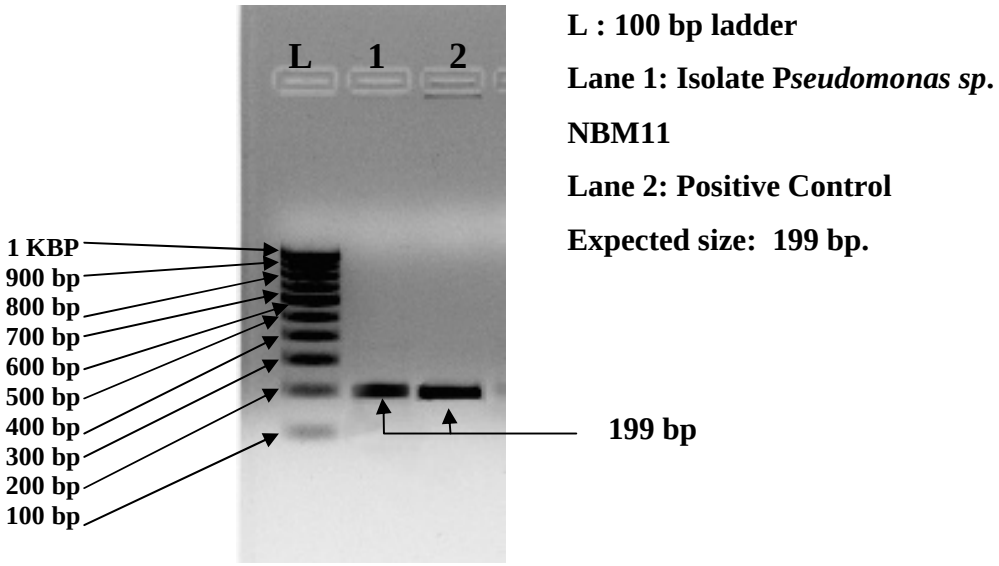

FIGURE 3.44: AMPLIFICATION OF PHENOL HYDROXYLASE GENE IN PSEUDOMONAS SP.NBM11.

The *DmpN* gene encodes a 60.523 KD polypeptide called phenol hydroxylase P3 component of the enzyme phenol hydroxylase, which is involved in the conversion of phenol to catechol. It is supposed to be induced by the primary substrate phenol (Selvaratanam et al., 1995). In the present study the microbes was screened with primer designed for the PCR amplification of the *DmpN* gene. Figure 3.44, suggests the amplification of a 199 bp band in the isolate *Pseudomonas sp.* NBM11 which implies that presence of the gene. Hence it can be concluded that the microbial strain secretes the phenol hydroxylase enzyme for the biodegradation activity and degrades phenol via metacleavage pathway described in chapter 1.

Chapter 4

CONCLUSION

Chapter 4

Conclusion

With urbanization and extensive industrialization, the pollution of the environment with man-made (synthetic) organic compounds has become a major problem. Huge quantity of waste water generated from human settlement and industrial sectors find their way to natural water bodies. The wastewater is enriched with varied pollutants and harmful both to human being and the aquatic flora and fauna and its successive accumulation in the soil has adverse effect on soil productivity. Phenol is a major pollutants being discharged from the effluents of various sources. They mix in the water bodies and make them unusable. Several physical and chemical methods for removal or treatment of phenol are in use. The physico-chemical removal or treatment technologies have been found to have inherent drawback owing to the tendency to form secondary toxic intermediates and also proven to be costly. Complete removal of the pollutants by the use of these processes is not possible. Hence biological method for degradation of phenol is adapted now days. Critical appraisal of the literature reveals that biological treatment is economical, practical and the most promising and versatile approach as it leads to complete mineralization of phenol producing non toxic end products. During the last four decades, considerable research efforts have been made to understand the biodegradation of the phenol and the genes responsible for encoding the enzymes involved in degradation pathways. Over the years, indigenous microbes isolated from the contaminated sites of industrial effluents and waste waters have been used for the study. The phenol contamination due to other sources like hospital waste has been overlooked by the scientific community which necessitates further understanding of the problems related to it. Thus, there exist many grey areas requiring further extensive fundamental studies in the field of biodegradation of phenol in newer sources other than the industries discharging phenol.

The work reported in this is an attempt to isolate a microorganism from the site contaminated with phenol from hospital sewage. Most of the disinfectant used today is phenol based. The sewage from the hospital contains phenol and contaminates the nearby

water bodies to which it is discharged as well as the soil where it accumulates. In the present study focused on the isolation of a microbe from soil collected from hospital sewage disposal site and its characterization has been carried out. The biodegradation potential of the microbe has been determined and the biodegradation potential of the microbe has compared with other known phenol degrading bacteria reported earlier. To understand the enhancement in the biodegradation potential, the microorganism has been immobilized in alginate beads and the biodegradation potential of the microbe on immobilization and in its freely suspended cellular form has been compared. The microbial strains subjected in this study were screened using specific primers designed from the available literature for the amplification of the gene or the component of the gene to evident the presence of the gene in the microbe to provide the possible reason and pathway for its biodegradation activity. The salient features of the findings were outlined below.

1. As many as 30 representative microorganisms were isolated from the phenol enriched soil samples, which were capable to grow on phenol as sole carbon source.
2. All the bacterial isolates, which were stable even at higher concentrations, were screened for their ability to grow and degrade phenol at 250 ppm concentration. Out of these isolates, only six were selected which yielded more than 80% phenol degradation and they were subjected to higher initial concentration of phenol like 500 ppm and 1000 ppm. Of the strains tested, NBM11 has shown a higher potential to degrade phenol at both 500 and 1000 ppm which ultimately led to higher biomass production by the strain.
3. At all these concentrations tested, NBM 11 has been found to be the most efficient phenol degrader and, hence, selected for further degradation studies. Based on the morphological and biochemical tests, it has been tentatively identified as *Pseudomonas* sp.
4. The process parameters were optimized. Effect of pH on phenol degradation by individual microorganism shows the rate of phenol degradation by *Pseudomonas sp.* NBM11 is maximum at pH 7. Influence of incubation temperature suggests the rate of phenol degradation by *Pseudomonas sp.* NBM11 is maximum at an optimum temperature from 30^0C - 32.5^0C.

5. The isolated microbe *Pseudomonas sp.* NBM11 from the contaminated soil sample is an efficient phenol degrading bacteria which is able to tolerate phenol up to a concentration as high as 1100 PPM. Under optimized condition the microorganism is able to degrade 1000 PPM of phenol completely in 168 hours.
6. Up to 500ppm initial phenol concentration, the isolated microbe has been found to have better degradation efficiency than other efficient phenol degrading microorganisms like *Pseudomonas putida* and *Acinetobacter calcoaceticus*. At higher initial phenol concentration like 750ppm and 1000 ppm, *Acinetobacter Calcoaceticus* was found to degrade phenol at a faster rate than the isolate but the degradation potential of the isolate is better than *Pseudomonas Putida* and other reference microbes.
7. With increase in the concentration of the phenol in the medium, the specific growth of the microorganism decreases which owes to the toxic nature of the substrate.
8. A severe increase in degradation potential of the microbes has been observed by immobilizing them in alginate bead. The isolated strain *Pseudomonas sp*. NBM11 is able to degrade 500 PPM of phenol in 84 hours as free cell form while on immobilizing the cells on alginate beads the degradation completes within 24hours. Similarly the strain utilizes 1000 ppm of phenol completely in 168 hours in its freely suspended cellular form while on immobilization it completely mineralizes the substrate in 48 hours. In general for all the microorganisms used in this study, on immobilization in calcium alginate bead, the degradation limit and rate has been found to be enhanced significantly
9. Immobilization of the microbes on solid matrix is an advantage over the freely suspended ones since they can be used repeatedly, making the treatment process with enhanced cost effective rate of degradation and tolerance to the toxic substrate.
10. Presence of a 199 bp band in amplified DNA indicated that the isolated bacterium is phenol- degrading because of having phenol hydroxylase gene.
11. Biodegradation activity of the microbe *Pseudomonas resinovorans* and *Arthrobacter Sps*. was attributed to the phenol hydroxylase gene LmpH which was observed by the presence of a 600 bp and 400 bp band amplicon from the DNA amplification using corresponding specific primers respectively.

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APPENDIX I

Mineral Salt Medium:

Composition	Quantity
K_2HPO_4	500 mg
KH_2PO_4	250 mg
NaCl	0.5g
NH_4SO_4	230 mg
$CaCl_2.2H_2O$	7.5 mg
$MgSO_47.H_2O$	100 mg
$MnSO_4.7H_2O$	100 mg
$FeCl_3$	1 mg
Double Distilled Water	1000 ml

Nutrient Media:

Composition	Quantity
Beef Extract	10g
Bacterial peptone	10g
NaCl	5g
Bacterial Agar	20g
Double Distilled Water	1000 ml

Nitrate Broth:

Composition	Quantity
Peptone	5g
Meat Extract	3g
Potassium Nitrate	1g
Double Distilled Water	1000ml
pH	7 ± 0.2

Reagent A: Dissolve 8g of Sulfanic Acid in 1 litre of 5N acetic acid. Store the reagent in dark amber bottle.

Reagent B: Dissolve 6g of N,N-Dimethyl-1-napthylamine in 1 litre of 5N acetic acid. Store the reagent in dark amber bottle in refrigerator.

MRVP Medium:

Composition	Quantity
Peptone	7g
Dextrose	5g
Dipotassium Phosphate	5g
Double distilled water	1000ml
pH	6.9±0.2

Carbohydrate Fermentation Broth:

Composition	Quantity
Carbohydrate to be tested	0.5-1.0%
Nutrient Broth	1000ml
Phenol Red	0.25g
pH	7.4

Gelatin Agar:

Composition	Quantity
Bactopeptone	5.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Gelatin	4.0 g
Agar	18.0 g
Distilled water	1000 ml
pH	7.0

Citrate Agar:

Composition	Quantity
Sodium Chloride	5g
Sodium Citrate	2g
Ammonium Dihydrogen Phosphate	1g
Dipotassium Phosphate	1g
Magnesium Sulfate	0.2g
Bromothymol Blue	0.08g
Agar	15g
Double Distilled Water	1000ml
pH	6.9±0.2

Preparation of Kovac's Reagent:

- 25ml of conc. HCL was added to75ml of Amyl alcohol.
- 5g of 4-Dimethylaminobenzenealdehyde was dissolved in the solution.
- The reagent was store at 4^{o}C in closed vials.

Preparation of Glycerol Stock:

1. The bacterial culture was inoculated overnight for preparation of the glycerol stock.
2. 700µL of the overnight grown culture was add to 300 µL of autoclaved glycerol and mixed properly and was immediately transferred to ice.
3. It was stored at -20°C for future use.

Preparation of TE Buffer:

- 100mM of Tris HCL (pH-8.0)+10mM EDTA(pH-8.0)
- To 1.21g of Tris Cl was dissolved with 0.372g of EDTA and the volume was made up to 100ml after adjusting the pH 8.0.

Preparation of Tris Saturated Phenol:

- Phenol was melted at 68^{o}C and hydroquinoline was added to a final concentration of 0.1%.
- Equal volume of 1M Tris-Cl was added and the mixture was added for 15 minutes.
- The upper aqueous layer was removed.
- The above two steps were repeated with the lower layers with 1M Tris (pH-8.6) and finally with 0.5M Tris (pH-8.6) for 2-3 times until the pH of the phenol reaches 8.0.
- 0.1M Tris (equal volume) of pH 8.0 was added to phenol containing 0.2% β-mercaptoethanol and stored in dark amber colored bottle at 4^{o}C.

Preparation of CTAB-NaCl Solution:

- To 4.1g of NaCl, 80ml of water and 10g of CTAB was added to it while heating and stirring continuously. It was heated up to 65^{o}C to dissolve and later the volume was adjusted to 100ml.

APPENDIX II

4-AMINO ANTIPYRENE METHOD OF PHENOL DETERMINATION

BUFFER SOLUTION

16.9g NH_4CL was dissolved in 143ml of Concentrated NH_4OH and diluted to 250 ml with double distilled water. The concentration of the buffer was such that 2ml of this buffer was able to adjust 100ml of distillate to pH 10.

AMINOANTIPYRINE SOLUTION

2 g of 4-aminoantipyrine was dissolved in distilled water and volume was made up to 100 ml.

POTASSIUM FERRICYANIDE SOLUTION

8 g of potassium ferricyanide was dissolved in distilled water and volume was made up to 100 ml.

PROCEDURE

- 100 mL of the sample or aliquot diluted to 100 mL, was added 2 mL of buffer solution and mixed properly. The pH of the sample should be 10 ± 0.2.
- 2.0 mL of 4-aminoantipyrine solution was added to the above mixture and mixed.
- 2.0 mL potassium ferricyanide solution was added to the above mixture and mixed.
- Absorbance at 510 nm was observed after 15 minutes.

APPENDIX III

(LIST OF INSTRUMENTS USED IN THE STUDY)

NAME OF THE INSTRUMENT	COMPANY
ORBITAL SHAKER INCUBATOR	WADEGATI
LAMINAR AIR FLOW HOOD	LABOTECH
SPECTRUM	SYSTRONICS
PH METER	SYSTRONICS
WEIGHING BALANCE	AFCOSET ER-200A
AUTOCLAVE	TOMY
WATER DISTILLATION UNIT	MILLIPORE
WATER BATH	ECOLINE
CENTRIFUGE	REMI
THERMOCYCLER	BIORAD
GEL DOCUMENTATION UNIT	BIORAD
ELECTROPHORETIC UNIT	BIORAD
SCANNING ELECTRON MICROSCOPY	JEOL JSM-6330F, JAPAN

Curriculum Vitae

Satyasundar Mohanty

Date of Birth: 21/04/1988

Email: satyasundarmohanty88@gmail.com

Permanent Address: Plot no. 171(P), Infront of Radha Krishna Mandir, Bayababa Lane, PO: Bhoi Nagar, Bhubaneswar, Odisha, 751022.

Education:

2012 M.Tech Dissertation Submitted
National Institute of Technology, Rourkela

2009 B.Tech (Biotechnology)
ICFAI University, Dehradun

List of Conference Papers:

1. Mohanty S. S., Jena H. M. & Satpathy G. R., 2011. "*Comparative study of biodegradation of phenol by different Pseudomonas sps.*" at World Congress on Biotechnology-2011, Hyderabad organized by OMICS Publishing group.

2. Mohanty S. S., Jena H. M. & Satpathy G. R., 2011. "*The Comparative Study of the Phenol Biodegradation Potential of Free Cells and Immobilized Pseudomonas Resinovorans*" at International Congress of Environmental Research -2011, SVNIT, Surat, Gujurat.

3. Mohanty S. S., Jena H. M. & Satpathy G. R., 2011. "*Determination of Phenol Utilization and Respiratory Activity by Phenol Degrading Arthrobacter Sps.*" in AICTE sponsored National Seminar on Management of Industrial Pollution (MIP – 2011) held at Purusottam Institute of Engineering and Technology, Rourkela, Odisha.

4. Mohanty S. S., Jena H. M. & Satpathy G. R., 2011. "*Phenol Biodegradation by Novel Pseudomonas Sps., Isolated from Phenol Contaminated Soil.*" at National Congress of Biotechnology – 2011, Orissa University of Agriculture Technology,Bhubaneswar, Odisha.